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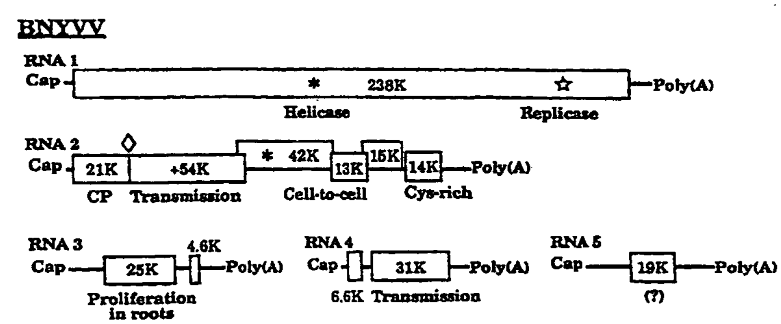

(57) Abstract

The present invention relates to a method for conveying resistance to beet necrotic yellow vein virus (BNYVV) to a sugar beet plant, which method comprises the following steps: (a) preparing a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV), (b) introducing said DNA fragment, operatively linked to a promotor that is active in sugar beet plants, into a sugar beet plant cell to obtain a transformed sugar beet cell; and (c) regenerating a transgenic sugar beet plant from the transformed sugar beet plant cell. In addition, the invention relates to a DNA vector harboring a fragment of a least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of BNYVV and transcription and translation regulatory sequences operably linked therewith, as well as to the use of said DNA vector for the transformation of a plant cell. The invention further relates to a plant cell comprising in its genome a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of BNYVV and the use of said plant cell for the regeneration therefrom of a sugar beet plant that is resistant against BNYVV.

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METHOD FOR CONVEYING BNYVV RESISTANCE TO SUGAR BEET PLANTS

The present invention relates to a method for conveying viral resistance to beet necrotic yellow vein virus (BNYVV) to a sugar beet plant. Furthermore, the invention relates to virus-resistant plants obtained according to this method, as well as to seeds and progeny derived therefrom.

Plant viruses are a serious problem for many of the major agricultural crops. Thus, BNYVV which is transmitted by the soilborne plasmodiophoromycete fungus *Polymyxa betae*, is the cause of the economically important disease of sugar beet (*Beta vulgaris*), known as rhizomania. This disease was first reported in Italy in the 1950s and has since become a major threat to sugar beet crops in most growing areas in the world.

Under field conditions BNYVV is generally confined to the subterranean portions of the plant. Infected sugar beet roots exhibit an increased proliferation and necrosis of the lateral roots, a decrease in the total mass of the main root and a consequent reduction in sugar yield. Plant death may also follow infection, particularly if the crop is infected early in season. Occasionally, the virus will ascend into the leaves where it can provide invasive vein-associated chlorotic and necrotic lesions.

BNYVV is a type member of the benyvirus family, which are fungus-transmitted, rod-shaped viruses possessing a divided genome composed of two or more components of single-stranded (ss) RNA. Four different plus sense ssRNA species of BNYVV have been identified, which are referred to as RNAs 1 to 4 in decreasing order of size (RNA 1: 6.8 kb; RNA 2: 4.7 kb; RNA 3: 1.8 kb; RNA 4: 1.5 kb). Some Japanese isolates also contain a fifth RNA component (RNA 5: 1.45 kb). All RNAs have been cloned and sequenced. The genetic map of the viral RNAs is shown in figure 1.

All five RNAs terminate in 3' poly-A tails, and RNAs 1-4 have cap structures at their 5' termini. RNAs 1

and 2 encode basic host-independent "housekeeping" functions, while the smaller RNAs intervene specifically in the natural infection process, including vector-mediated infection of sugar beet roots, proliferation within the root system and production of rhizomania symptoms. Thus, RNA 1 has been shown to code for viral RNA polymerase activity and RNA 2 encodes the 21-kd viral coat-protein. The 3'-proximal half of RNA 2 carries a group of three successive slightly overlapping viral genes, known as the triple gene block (TGB3), that are closely similar to a cluster of three genes in other rod-shaped plant viruses, and are involved in cell to cell movement of the virus. RNA 3 is associated with massive proliferation of fine rootlets in sugar beet and facilitates the spread of the virus in root tissue, whereas RNA 4 increases the efficiency of transmission of the virus by the fungus.

Fungus-transmitted viruses, such as BNYVV may be retained in resting spores in soil for years once a field becomes infested. As there exist no effective chemical or physical methods for eliminating the virus, neither in the plants nor in the soil, the only option for the sugar beet farmer is the use of genetically resistant cultivars. Several companies have provided a number of tolerant, even partially resistant varieties, by transferring wild type tolerance genes into commercially varieties through breeding. This is, however, a very tedious and time-consuming process, generally taking a long time before useful resistant plants are obtained. In addition, under high disease pressure tolerant or partially resistant plants will develop disease symptoms because the resistance level is too low. Another problem with tolerant or only partially resistant plants lies in the fact that virus populations continue to grow resulting in the possible emergence of a BNYVV strain which may break the resistance/tolerance genes.

The rapid revolution in the areas of plant engineering has led to the development of new strategies to confer genetic resistance to viruses. Resistance to viral diseases through the introduction of portions of viral genome sequences has become a new source of resistance. The viral sequence (construct) is transformed into a plant by combining the use of appropriate cell or tissue culture techniques and a DNA delivery system such as the well known Agrobacterium tumefaciens transformation system or the direct DNA transfer mediated by chemicals like polyethylene glycol (PEG).

Known methods of inducing pathogen defense mechanisms in plants are for example described in EP 9687106.0, which relates to a method for inducing resistance to a virus comprising a TGB3 sequence. The publication describes that it was possible to induce BNYVV resistance by a method, which consists of transforming plant cells with a DNA construct corresponding to a fragment of the nucleic acid sequence of the genomic or subgenomic RNA 2 of the BNYVV. The disadvantage of this method is however, that only tolerance is obtained, and not resistance. In tolerant host plants there can still be normal levels of plant virus replication, but the plant shows little or no visible signs of infection, whereas in resistant hosts the virus replication is low or even absent.

In WO 93/25068 viral resistance in plants is induced by plant transformation with a replicase portion (RNA 1) of a plant virus genome. This publication does not refer to sugar beet, nor to BNYVV.

Sugar beet are known to be recalcitrant species in genetic engineering, complicating successful induction of BNYVV resistance. However, because of the extent and impact of the viral disease of BNYVV there is a major interest to improve the sources of genetic resistance in sugar beet plants.

The object of the present invention is, therefore, to provide means to obtain sugar beet plants,

that exhibit resistance to BNYVV. Preferably, they exhibit total resistance or immunity, by combining different approaches and by using methods which do not allow the virus to replicate.

This is achieved by the invention by providing a method for conveying resistance to beet necrotic yellow vein virus (BNYVV) to a sugar beet plant, comprising the following steps:

(a) preparing a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV),

(b) introducing said DNA fragment, operately linked to a promotor that is active in sugar beet plants, into a sugar beet plant cell to obtain a transformed sugar beet cell; and

(c) regenerating a transgenic sugar beet plant from the transformed sugar beet plant cell.

Thus, a sugar beet plant is obtained which exhibits a durable resistance and in which the virus does not replicate. This is a unique aspect of the transformant of the invention and has not been disclosed before.

According to the invention the essential sequence homology of the fragment is a homology of at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

The homology or "degree of similarity" is used to denote nucleotide sequences which when aligned have similar (identical or conservatively replaced) nucleotides in like positions or regions. For example, two nucleotide sequences with at least 85% homology to each other have at least 85% homologous (identical or conservatively replaced nucleotides) in a like position when aligned optimally allowing for up to 3 gaps, with the provison that in respect of the gaps a total of not more than 15 amino acid resides is affected. The degree of similarity may be determined using methods well known

in the art (see, for example, Wilbur, W.J. and Lipman, D.J. "Rapid Similarity Searches of Nucleic Acid and Protein Data Banks." Proceedings of the National Academy of Sciences USA 80, 726-730 (1983) and Myers E.and Miller W. "Optimal Alignments in Linear Space". Comput. Appl. Biosci. 4:11-17(1988)). One programme which may be used in determining the degree of similarity is the MegAlign Lipman-Pearson one pair method (using default parameters) which can be obtained from DNAstar Inc, 1228, Selfpark Street, Madison, Wisconsin, 53715, USA as part of the Lasergene system. The test for homology of the sequence is based on the percent identity which is calculated by Fast DB based on the following parameters: mismatch penalty 1.0, gap penalty (1.00), gap size penalty 0.33 and joining penalty 30.0.

Preferred embodiments of the invention use various fragments having nucleic acid sequences that correspond with the homology indicated or completely to nucleotides 153 to 3258, 169 to 539, 1226 to 1683, 2754 to 3192 or to all of the 6746 nucleotides of RNA 1.

The present invention also includes DNA which hybridises to the DNA of the present invention and which codes for RNA1. Preferably, such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3 x SCC at about ambient temperature to about 65°C, and high stringency conditions as 0.1 x SSC at about 65°C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SSC is three time as strong as SSC and so on.

The fragment can be introduced into a regenerable plant cell by means of a DNA vector harboring the fragment and transcription and translation regulatory sequences operably linked therewith using standard plant transformation methods such as Agrobacterium-mediated transformation of cells embedded in plant tissues such as cotyledons (Krens et al., Plant Science 116: 97-106; 1996) or polyethylene glycol-mediated DNA uptake of

single cells like the guard cell protoplasts (Hall et al., Nature Biotechnology 14: 1133-1138; 1996). The DNA vector harboring the fragment is also part of the present invention.

The use of constructs constructed such that the gene sequence inhibits or promotes gene expression is quite well understood. A complete gene sequence, under the control of a promoter that operates effectively in the plant, will generally overexpress the gene product, leading to an amplification of the effect of the protein so produced. Sometimes the gene product is reduced: this phenomenon is termed "co-suppression". Downregulating this gene can be done by several techniques. It can be done by 'dominant-negative' constructs. These contain the specific DNA binding domain as well as possible dimerisation domains but are transcriptionally inactive. They 'sit' on the promoters of the target genes and thereby prevent the binding of the endogenous protein. Additionally, reduction of the gene product can also be obtained by using such dominant negative mutation, or by reversing the orientation of the gene sequence with respect to the promoter so that it produces a type of gene product called "antisense" messenger RNA.

A DNA construct according to the invention may be an "antisense" construct generating "antisense" RNA or a "sense" construct (encoding at least part of the functional protein) generating "sense" RNA.

"Antisense RNA" is an RNA sequence which is complementary to a sequence of bases in the corresponding mRNA: complementary in the sense that each base (or the majority of bases) in the antisense sequence (read in the 3' to 5' sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5' to 3' sense. Such antisense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged to generate a transcript with at least part of its sequence complementary to at least part of the coding strand of

the relevant gene (or of a DNA sequence showing substantial homology therewith).

"Sense RNA" is an RNA sequence, which is substantially homologous to at least part of the corresponding mRNA sequence. Such sense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged in the normal orientation so as to generate a transcript with a sequence identical to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). Suitable sense constructs may be used to inhibit gene expression (as described in International Patent Publication WO91/08299).

DNA constructs according to the invention may comprise a base sequence at least 10 bases (preferably at least 35 bases) in length for transcription into RNA. There is no theoretical upper limit to the base sequence - it may be as long as the relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences between 100 and 1000 bases in length. The preparation of such constructs is described in more detail below.

As a source of the DNA base sequence for transcription, a suitable cDNA or genomic DNA, RNA or synthetic polynucleotide may be used. The transcriptional initiation region (or promoter) operative in plants may be a constitutive promoter (such as the 35S cauliflower mosaic virus promoter) or an inducible or developmentally regulated promoter, as circumstances require. Suitable DNA sequences for control of expression of the plant expressible genes (including marker genes), such as transcriptional initiation regions, enhancers, leader sequences, non-transcribed leaders and the like, may be derived from any gene that is expressible in a plant cell. Also hybrid promoters combining functional portions of various promoters, or synthetic equivalents thereof can be employed. Apart from constitutive promoters,

inducible promoters, or promoters otherwise regulated in their expression pattern, e.g. developmentally or cell-type specific, may be used to control expression of the expressible genes according to the invention. For example, it may be desirable to modify protein activity at certain stages of the plant's development. Use of a constitutive promoter will tend to affect protein levels and functions in all parts of the plant, while use of a tissue-specific promoter allows more selective control of gene expression and affected functions.

Another option under this invention is to use inducible promoters. Promoters are known which are inducible by pathogens, by stress, by chemicals and by environmental signals. The induction of the gene activity by internal or external induction is within the scope of the present invention. Promoters of this type enable the inducibility of the gene activity in a controlled manner, thus the plant can develop normally without any undue influence by the transgene gene. Promoters that are inducible promoters include those described in DE 4446342 (fungus and auxin inducible PRP-1), WO 96/28561 (fungus inducible PRP-1), EP 0 712 273 (nematode inducible), EP 0 330 479 and US 5,510,474 (stress inducible), WO/96/12814 (cold inducible), and Zeneca's alcohol inducible promoter. Other inducible promoters are described in EP 0 494 724, EP 0 619 844, WO 92/19724. Thus the gene product, whether antisense or sense RNA or the peptide, is only produced in the tissue at the time when its action is required.

As mentioned above, the term "inducible promoter" includes promoters which may be induced chemically. The use of a promoter sequence which is controlled by the application of an external chemical stimulus is most especially preferred. The external chemical stimulus is preferably an agriculturally acceptable chemical, the use of which is compatible with agricultural practice and is not detrimental to plants or mammals. The inducible promoter region most preferably

comprises an inducible switch promoter system such as, for example, a two component system such as the alcA/alcR gene switch promoter system described in the published International Publication No. WO 93/21334, the ecdysone switch system as described in the International Publication No. WO 96/37609 or the GST promoter as described in published International Patent Application Nos. WO 90/08826 and WO 93/031294, the teachings of which are incorporated herein by reference. Such promoter systems are herein referred to as "switch promoters". The switch chemicals used in conjunction with the switch promoters are agriculturally acceptable chemicals making this system particularly useful in the method of the present invention.

The skilled person will be capable of selecting a DNA vector for use in these methods. An example of a suitable vector for the Agrobacterium-mediated transformation is pBIN19. Suitable vectors for the PEG-mediated transformation include the pBluescript vector or pIGPD7 (Hall et al., Nature Biotechnology 14: 1133-1138; 1996). Introduction of the fragment into these vectors can be achieved by means of standard molecular biological techniques as for example described in Sambrook et al., "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory Press; 1989.

Transformed plants obtained by the method of the present invention show absolute resistance, or immunity, to BNYVV. In contrast, previous attempts to convey resistance to plants to BNYVV (Kallerhof et al., Plant Cell Reports 9: 224-228, 1990; and Mannerlof et al., Euphytica 90: 293-299, 1996) or other viruses, such as to tobacco mosaic virus (TMV) (Donson et al., Mol. Plant-Microbe Interact. 6: 635-642; 1993) by transforming plants with portions of the viral genome were less successful. Inoculated leafs still showed symptoms of infection, thus indicating that the resistance is not absolute. It is therefore surprising that the method of

the invention is capable of conveying absolute resistance to BNYVV to sugarbeet plants.

Furthermore the invention relates to a transformed plant cell and a transgenic plant resistant to BNYVV as well as reproducible structures, such as seeds, calluses, buds, embryos, obtained from the transgenic plants and the progeny derived therefrom.

In a preferred embodiment of the invention the resistance described herein can be combined with other types of resistance or tolerance to BNYVV.

The invention will further be illustrated in the following examples and figures, but is not limited thereto.

Figure 1 shows a diagrammatic representation of the genomic organisation of Beet Necrotic Yellow Vein Virus (based on Jupin et al., Seminars in Virology vol 2.2: 112-129; 1991).

Figure 2 shows the physical maps of pVDH239 and pVDH240. LB=left border, RB=right border, P35S=CaMV 35S promoter, NPTII= neomycin phosphotransferase II, T35S=CaMV 35S polyadenylation signal, GUSINT=beta-glucuronidase gene, BNYVVpolTRUNC=BNYVV cDNA1 fragment, Tnos=nopaline synthase gene derived polyadenylation signal. The positions of the main restriction enzyme recognition sites are indicated.

Figure 3 shows a Southern blot analysis by which the number of T-DNA insertions integrated into the genome of the primary sugar beet transformant T157-01 has been determined. The outline of the T-DNA structure of the binary vector pVDH239 is shown at the top.

Figure 4 shows diagrams of the individual ELISA values of the root extracts of sugar beet plants of the populations Cadyx (susceptibel control), Rifle (rhizomania tolerant variety), Rhizor (rhizomania tolerant variety) and T157-01 (GUS-positive F1 individuals) after inoculation with BNYVV-infested soil. Each number at the horizontal axis represents an individual plant.

Figure 5B shows a Southern blot analysis by which the number of T-DNA insertions integrated into the genome of the F1 progeny plants of T157-01 has been determined as well as a diagram of the individual ELISA values of the root extracts of the F1 progeny plants of T157-01) after inoculation with BNYVV-infested soil (figure 5A). The numbers on top of the Southern blots represent the lab-codes of the individual F1 progeny plants. The ELISA values indicated by "Genotype 1" in the lower pannel correspond to the indivduals showing a single band in the Southern blot (2012, 2019, 2021, 2029, 2030, 2031, 2034, 2035, 2038, 2042, 2044, 2046, 2051, 2052, 2061, 2066, 2068, 2069), whereas the ELISA values indicated by "Genotype 2 + 3" in the lower pannel correspond to the individuals showing 2 or 3 bands in the Southern blot (1999, 2000, 2001, 2007, 2008, 2011, 2013, 2014, 2015, 2016, 2017, 2018, 2020, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2032, 2033, 2036, 2037, 2039, 2040, 2041, 2043, 2045, 2047, 2048, 2049, 2050, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2062, 2063, 2064, 2065, 2067, 2070). The ELISA values indicated by "GUS(-) segregants" in the lower pannel correspond to the individual F1 progeny plants which are GUS-negative (1997, 1998, 2002, 2004, 2005, 2006, 2009, rest not shown).

EXAMPLES

EXAMPLE 1

Preparation of contruct with truncated BNYVV replicase sequence

Two primer combinations were used to obtain the cDNA clones of BNYVV for cloning in the tranformation vector (Bouzoubaa et al. J. Gen. Virol. 68:615-626; 1987).

For the 5'-end the primers were

P1: 5'-CGCGGATCCACCATGGCAGATTCGTTC-3' (containing a BamHI and NcoI restriction site and nucleotides identical to nucleotides 153-168), and

P2: 5'-GACGAATTCAAGTCGTCTTTC-3' (EcoRI restriction site and nucleotides complementary to nucleotides 288-301). For the 3'-end the primers were

P3: 5'-GACGAATTCGAAAGATGAGTCTA-3' (EcoRI site and nucleotides identical to nucleotides 2799-2812), and

P4: 5'-CGCAGATCTTTAACTGCTCATCACCAAC-3' (BglII site and nucleotides complementary to nucleotides 3244-3258 and stop codon).

Before cloning the fragments into the Bluescript vector (Stratagene, La Jolla, CA, USA) the SalI/HincII/AccI site was replaced by a BglII site. After amplification of BNYVV cDNA using P1 and P2 a DNA fragment was obtained which was digested with BamHI and EcoRI and inserted into the modified, BamHI/EcoRI cleaved pBleuscript vector to yield pKSNBPol1. Subsequently, BNYVV cDNA1 was amplified using P3 and P4. The resulting DNA fragment was cleaved by EcoRI and BglII and inserted into pKSBNPol1 cleaved with EcoRI and BglII, yielding pKSNBPpol2.

Thereafter the AccI fragment of BNYVV cDNA1 identical to nucleotides 250-2815 was cloned in AccI cleaved pKSBNPol2. Thus, a pKSBNPoltrunc was obtained which comprised the BNYVV cDNA1 fragment (poltrunc) identical to the nucleotides 153-3258, flanked by a BamHI site at the 5'-end and by a BglII fragment at the 3'-end. The poltrunc fragment was cloned as BamHI-BglII fragment in the BamHI site of pVDH4 such that a functional sense poltrunc fragment was placed behind the CaMV 35S promotor and before the nopaline terminator sequence. The complete construct, carrying the CaMV35S promotor, the poltrunc fragment and nopaline terminator sequence was excised from the plasmid with ClaI and cloned in the binary transformation vector pVDH212 in which the BamHI site was converted into a ClaI site by inserting a molecular linker.

pVDH212 is a pBIN19 derived binary transformation vector, which contains 35S-NPTII (neomycin phosphotransferase under control of the CaMV 35S promotor as a selectable marker gene conferring resistance to kanamycin), as well as 35S-GUSi (gene encoding beta-glucuronidase (Vancanneyt et al., Mol. Gen. Genet. 220: 245-250; 1990). Thus, two different transformation vectors containing the poltrunc construct were obtained: one, pVDH239, with the poltrunc construct in the same transcriptional direction as the NPTII and GUS gene, and another, pVDH240, with the poltrunc construct having an opposite transcriptional direction. The binary transformation vectors pVDH239 and 240 (figure 2) were conjugated and transferred to Agrobacterium tumefaciens LBA4404 (Phabagen Collection, Utrecht, the Netherlands) resulting in HAT1239 (for pVDH239) and HAT1240 (for pVDH240) strains. HAT1239 and HAT1240 were used for transformation of sugar beet.

EXAMPLE 2

Transformation of sugar beet

Methods for Agrobacterium-mediated transformation using binary vectors are well established and known to a person skilled in the art.

To obtain transformed sugar beet plants, O-type B8M5.9 plants were transformed according to Krens et al. (Plant Science 116: 97-106; 1996). Transformation with Agrobacterium HAT1239 yielded 8 transformants: T156-03, T156-06, T156-09, T156-10, T156-13, T156-20, T150-30, and T157-01, and with Agrobacterium HAT1240 12 transformants were obtained: T183-01, T183-02, T183-06, T183-10, T183-16, T183-19, T183-21, T183-23, T183-26, T184-02, T184-03 and T184-04. The transformants were induced to flowering by vernalisation and used to produce seeds either through self-pollination (selfing or S1 seed) or cross-pollination on male sterile plants (hybrid or F1 seed). The pollination was performed in closed pollen chambers

to prevent pollination by sugar beet pollen from a different source.

The F1 seeds were used as starting material to carry out a bioassay for BNYVV resistance. The seedlings were screened initially for GUS activity which indicates the presence of the T-DNA. GUS-negative segregants were used as a negative control in the bioassay. Depending on the total number of T-DNA inserts, the resistance, if present, can segregate within the GUS-positive population. Only T157-01 derived progeny showed BNYVV resistance.

Subsequently, the primary transformant T157-01 was analysed in further detail on Southern blot. Genomic DNA was isolated from leaves, digested seperately with EcoR1, BamHI and SacI and used to prepare a Southern blot which was subsequently probed with GUS. From this experiment it was concluded that the primary transformant contained three T-DNA inserts (figure 3).

EXAMPLE 3

Bioassay

A lateral root bioassay was developed as a single plant test for the selection of rhizomania resistant sugarbeet plants. One-week-old plantlets were transplanted in a standard mixture of 10% rhizomania infected soil and further incubated for 4 weeks. The conditions for infection were optimised and standardised on the level of the infection pressure and the growth of rootlets (only formation of hairy roots). Incubation conditions were: 18.9-19^{0}C day/night, 70% relative humidity, 10.000 lux white light, pots at 32.5 cm from the lamps.

The amount of virus in the rootlets which was directly correlated with the resistance mechanism in the plant was measured by an ELISA method. The cut-off value for resistant plants was put at an OD-value of 0.2 (Clark et al. J. Virol. Meth. 15:213-222; 1987). Four weeks

after transplantation the lower part of the roots was used for ELISA. The root piece was dried on a filter paper and transferred to a mortar. Extraction buffer was added at a ratio of 10 volume equivalents of the root weight (dilution 1/10). Root juice was extracted by hand crushing. The juice was transferred to 1.5 ml tubes and centrifuged (300 rpm, 10 minutes) the supernatants were kep on ice and assayed. Populations tested were:

Population	Number of plants	Remarks
T 157-01 (F1)	73	GUS(+) individuals
Cadyx F 052	26	sensitive control
Rhizor F 202	27	tolerant control
Rifle	28	tolerant control

Within the group of T157-01 GUS(+) plants two categories can be observed (figure 4). One category displays immunity to BNYVV (ELISA cut-off 0.2) with an average ELISA value of 0.006 which is the background level of the experimental system. The other category shows normal susceptibility with an average ELISA value in the range of the susceptible control.

The infection pressure in this bioassay was high due to the increase of temperature during a part of the infection period. Consequently there was no clear distinction between Cadyx and Rizor/Rifle (figure 4). On the other hand, the resistant plants selected in the T 157-01 F1 progeny could be considered as totally resistant independent of the rhizomania infection pressure (figure 4). In conclusion, the introduced construct containing the BNYVV cDNA1 fragment resulted in a dramatic negative effect on the multiplication of BNYVV in the lateral roots of the inoculated transgenic sugar beet plants which effectively rendered the plants completely resistant to rhizomania.

In order to explain the segregation of the GUS(+) population in a resistant and a suceptible category the individual plants were analysed in a separate experiment for resistance to BNYVV and simultaneously for the presence of the T-DNAs by Southern analysis using SacI as restriction enzyme and GUS as a probe. The results, as depicted in Figure 5A and 5B, point out that the GUS(+) plants within this population which contained a single band on the Southern blot were all susceptible (average ELISA value 0.63), whereas the GUS(+) plants which contained either 2 or 3 bands on the Southern blot were all resistant (average ELISA value: 0.21). The GUS(-) segregants were all susceptible (average ELISA value 0.80). Apparently the resistant phenotype is linked to the presence of the upper and lower band of this particular band pattern obtained on Southern blot, whereas the presence of the middle band is not linked to the resistant phenotype. It is therefore concluded that the segregation of the GUS(+) population into a suseptible and a resistant category can be explained by the fact that one of the T-DNAs which can result in a GUS(+) phenotype does not contribute to the resistance to BNYVV.

CLAIMS

1. Method for conveying resistance to beet necrotic yellow vein virus (BNYVV) to a sugar beet plant, comprising the following steps:

(a) preparing a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV),

(b) introducing said DNA fragment, operately linked to a promotor that is active in sugar beet plants, into a sugar beet plant cell to obtain a transformed sugar beet cell; and

(c) regenerating a transgenic sugar beet plant from the transformed sugar beet plant cell.

2. Method as claimed in claim 1, wherein the essential sequence homology of the fragment is a homology of at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

3. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 153 to 3258 of RNA 1 of said virus.

4. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 169 to 539 of RNA 1 of said virus.

5. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 1226 to 1683 of RNA 1 of said virus.

6. Method according to claim 1 or 2 characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in

claims 1 and 2 to nucleotides 2754 to 3192 of RNA 1 of said virus.

7. Method according to claim 1 or 2 characterized in that the fragment consists of 6746 nucleotides.

8. Method as claimed in claims 1-7 characterized in that the fragment is introduced into the cell by means of a DNA vector harboring the fragment and transcription and translation regulatory sequences operably linked therewith.

9. DNA vector harboring a fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the <u>b</u>eet <u>n</u>ecrotic <u>y</u>ellow <u>v</u>ein <u>v</u>irus (BNYVV), and transcription and translation regulatory sequences operably linked therewith.

10. DNA vector as claimed in claim 9, wherein the essential sequence homology of the fragment is a homology of at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

11. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 153 to 3258 of RNA 1 of said virus.

12. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 169 to 539 of RNA 1 of said virus.

13. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 1226 to 1683 of RNA 1 of said virus.

14. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in

claims 9 and 10 to nucleotides 2754 to 3192 of RNA 1 of said virus.

15. DNA vector according to claim 9 or 10, characterized in that the fragment consists of 6746 nucleotides.

16. Use of a DNA vector as claimed in claims 9-15 for the transformation of a plant cell.

17. Plant cell comprising in its genome a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV).

18. Plant cell as claimed in claim 17, wherein the essential sequence homology of the fragment is a homology of at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

19. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 153 to 3258 of RNA 1 of said virus.

20. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 169 to 539 of RNA 1 of said virus.

21. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 1226 to 1683 of RNA 1 of said virus.

22. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 2754 to 3192 of RNA 1 of said virus.

23. Plant cell according to claim 17 or 18, characterized in that the fragment consists of 6746 nucleotides.

24. Plant cell as claimed in claims 17-23 being part of a sugar beet plant that is resistant against BNYVV.

25. Use of a plant cell as claimed in claims 17-23 for the regeneration therefrom of a sugar beet plant that is resistant against BNYVV.

26. Sugar beet plant consisting at least partly of plant cells as claimed in claims 17-23.

27. Progeny of a sugar beet plant as claimed in claim 26.

28. Seeds of a sugar beet plant as claimed in claim 26.

29. Vegetatively reproducible structures, such as calluses, buds, embryos, from a plant according to claim 26 or progeny according to claim 27.

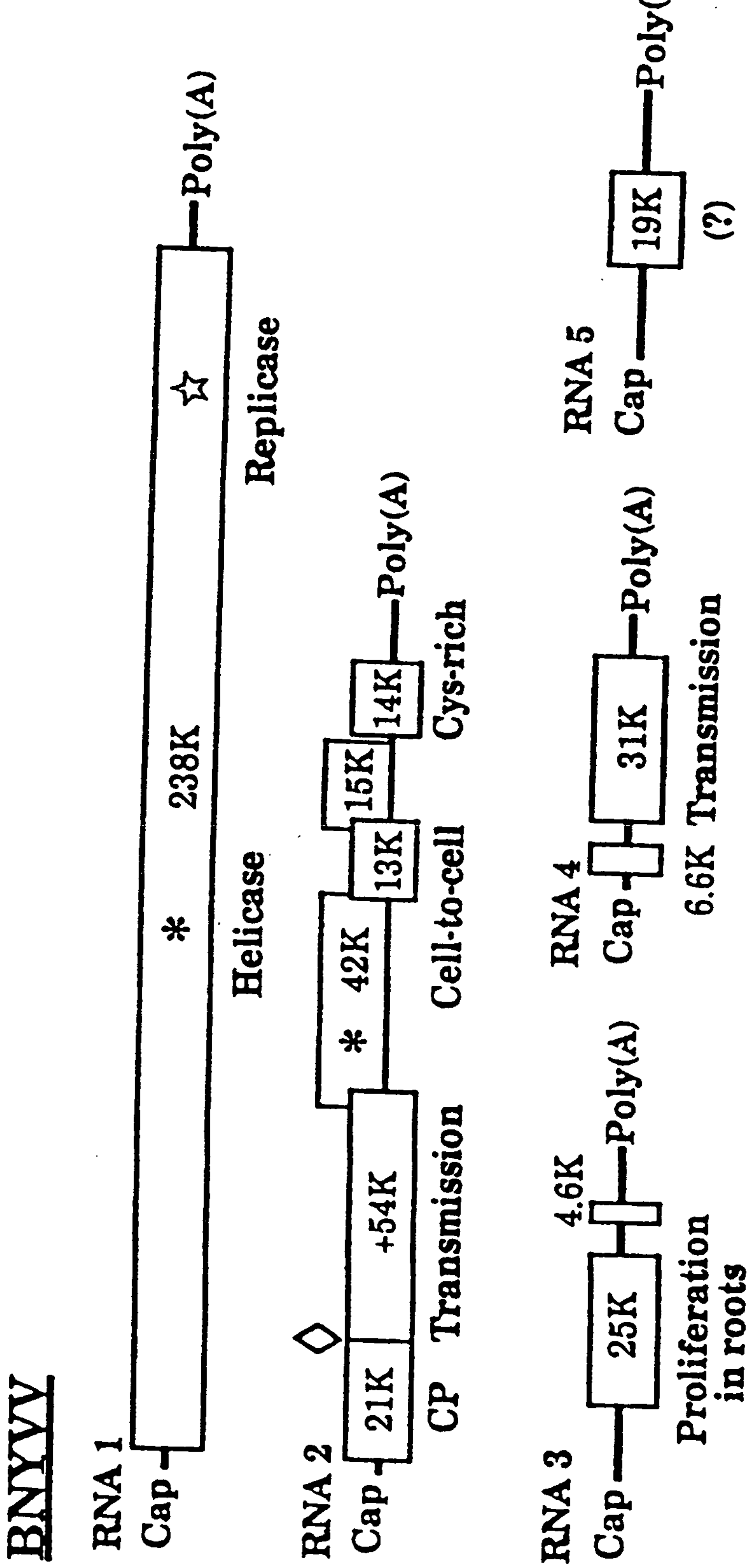

BNYVV
RNA 1
Cap
238K
Poly(A)
Helicase
Replicase
RNA 2
Cap
21K
+54K
42K
13K
15K
14K
Poly(A)
CP
Transmission
Cell-to-cell
Cys-rich
RNA 3
Cap
25K
4.6K
Poly(A)
Proliferation
in roots
RNA 4
Cap
6.6K
31K
Poly(A)
Transmission
RNA 5
Cap
19K
(?)
Poly(A)

FIG. 1

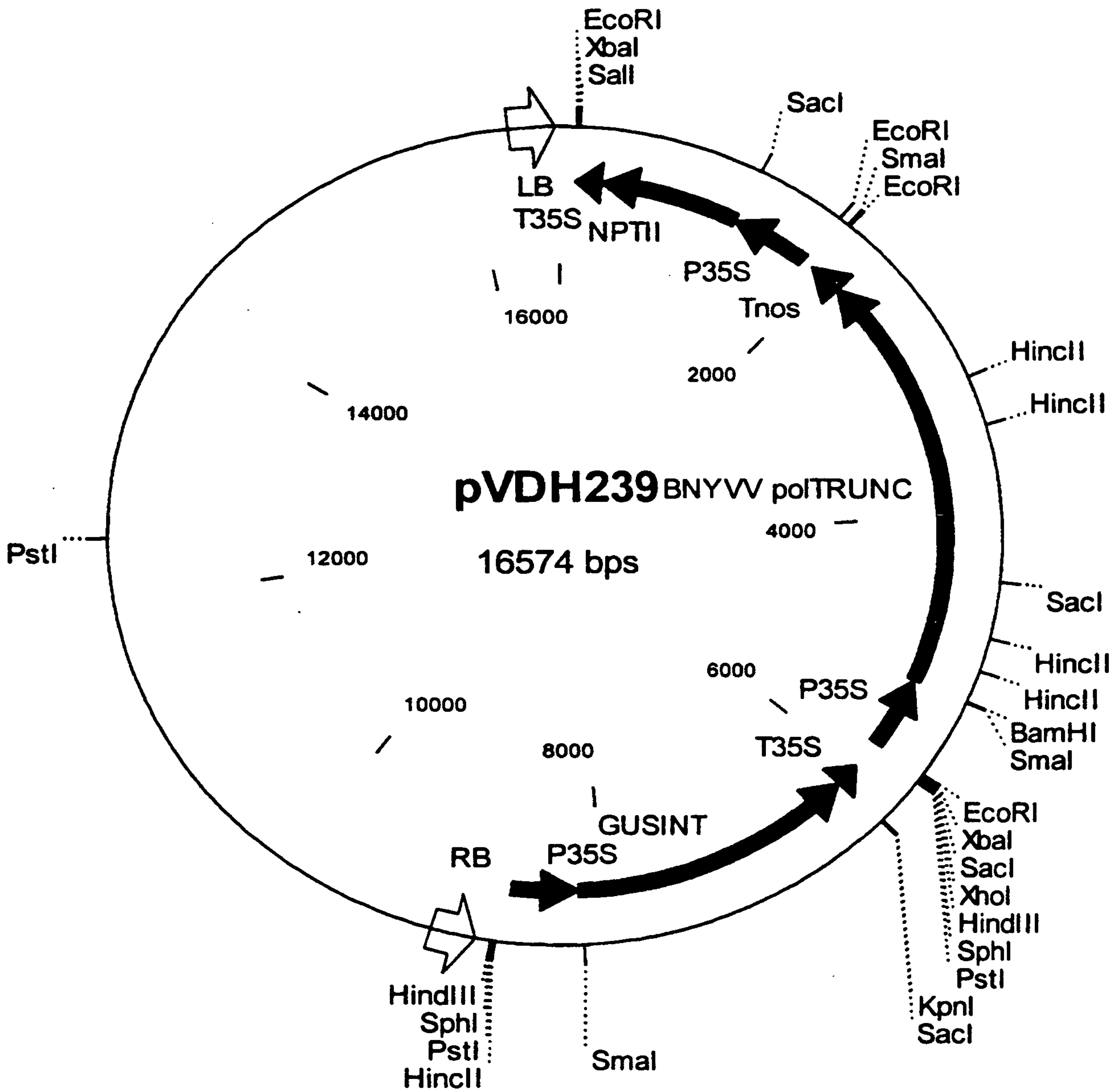
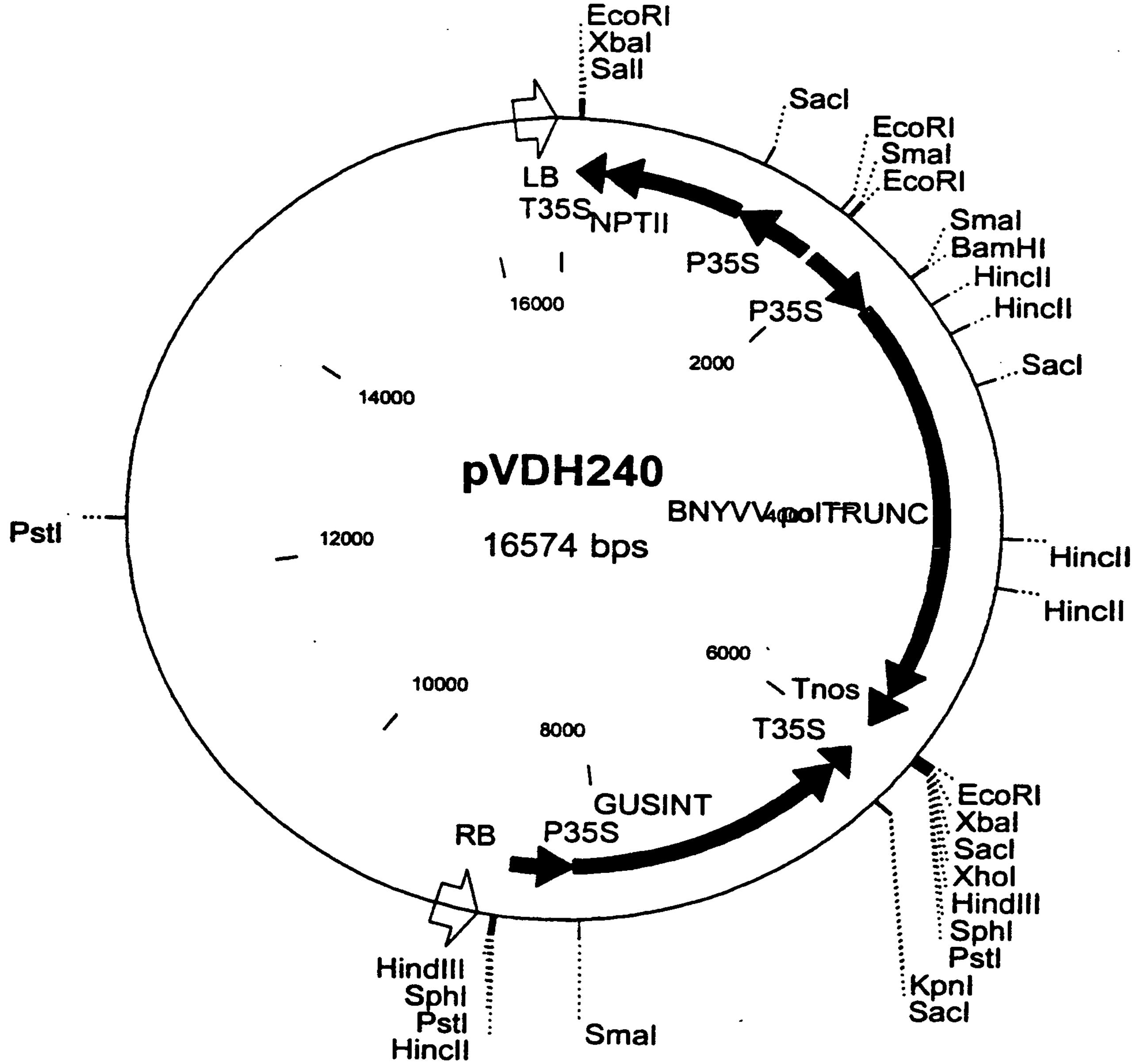

EcoRI
XbaI
SalI
SacI
EcoRI
SmaI
EcoRI
LB
T35S
NPTII
P35S
Tnos
16000
2000
HincII
HincII
14000
pVDH239 BNYVV polTRUNC
4000
PstI
12000
16574 bps
SacI
HincII
6000
HincII
P35S
10000
BamHI
SmaI
8000
T35S
EcoRI
XbaI
GUSINT
SacI
RB
P35S
XhoI
HindIII
SphI
PstI
KpnI
SacI
HindIII
SphI
PstI
HincII
SmaI

FIG. 2A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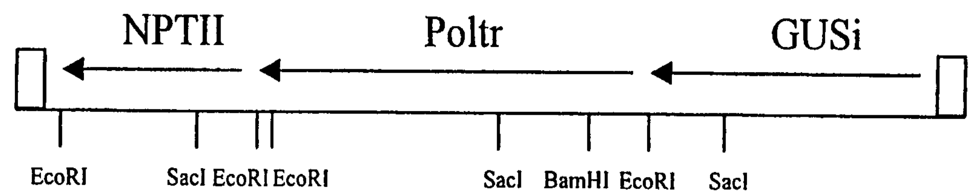
XbaI
SalI
SacI
EcoRI
SmaI
EcoRI
SmaI
BamHI
HincII
HincII
SacI
LB
T35S
NPTII
P35S
P35S
16000
2000
14000
pVDH240
16574 bps
BNYVVpolTRUNC
PstI
12000
HincII
HincII
6000
Tnos
10000
T35S
8000
GUSINT
RB
P35S
EcoRI
XbaI
SacI
XhoI
HindIII
SphI
PstI
KpnI
SacI
HindIII
SphI
PstI
HincII
SmaI

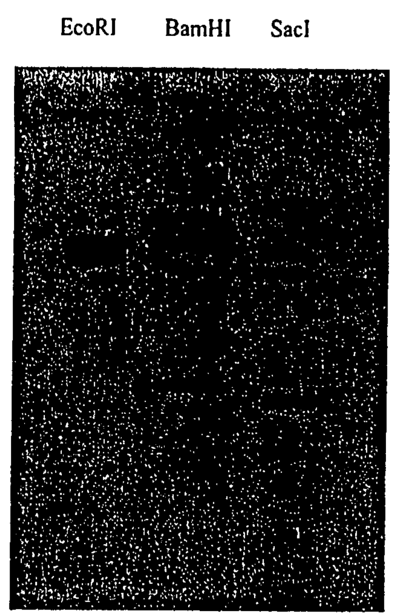
FIG. 2B

Southern analysis sugar beet transformant T157-01 (pVDH239)

T-DNA:



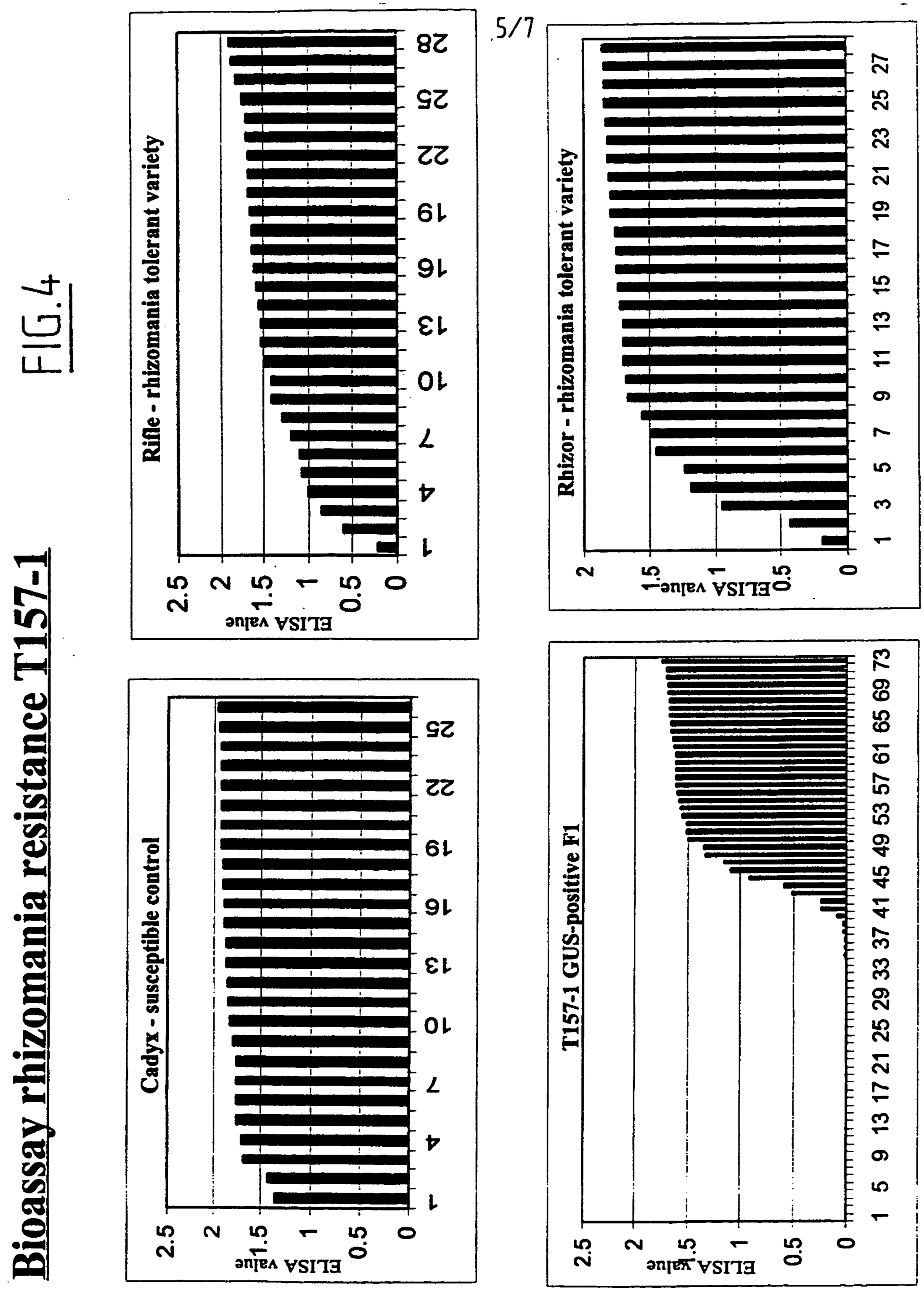
Probe: GUSi



Conclusion: T157-01 contains 3 inserts

FIG. 3

5/7


Bioassay rhizomania resistance T157-1
FIG.4
Rifle - rhizomania tolerant variety
Cadyx - susceptible control
Rhizor - rhizomania tolerant variety
T157-1 GUS-positive F1
ELISA value

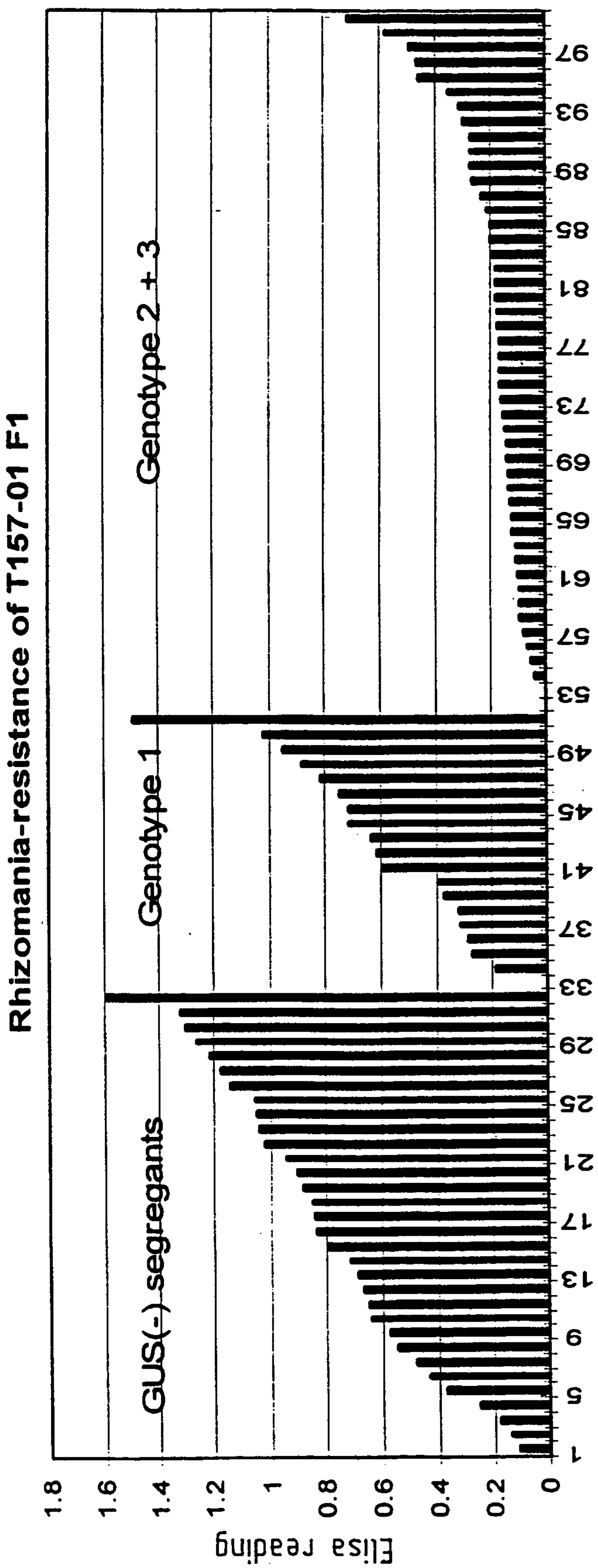
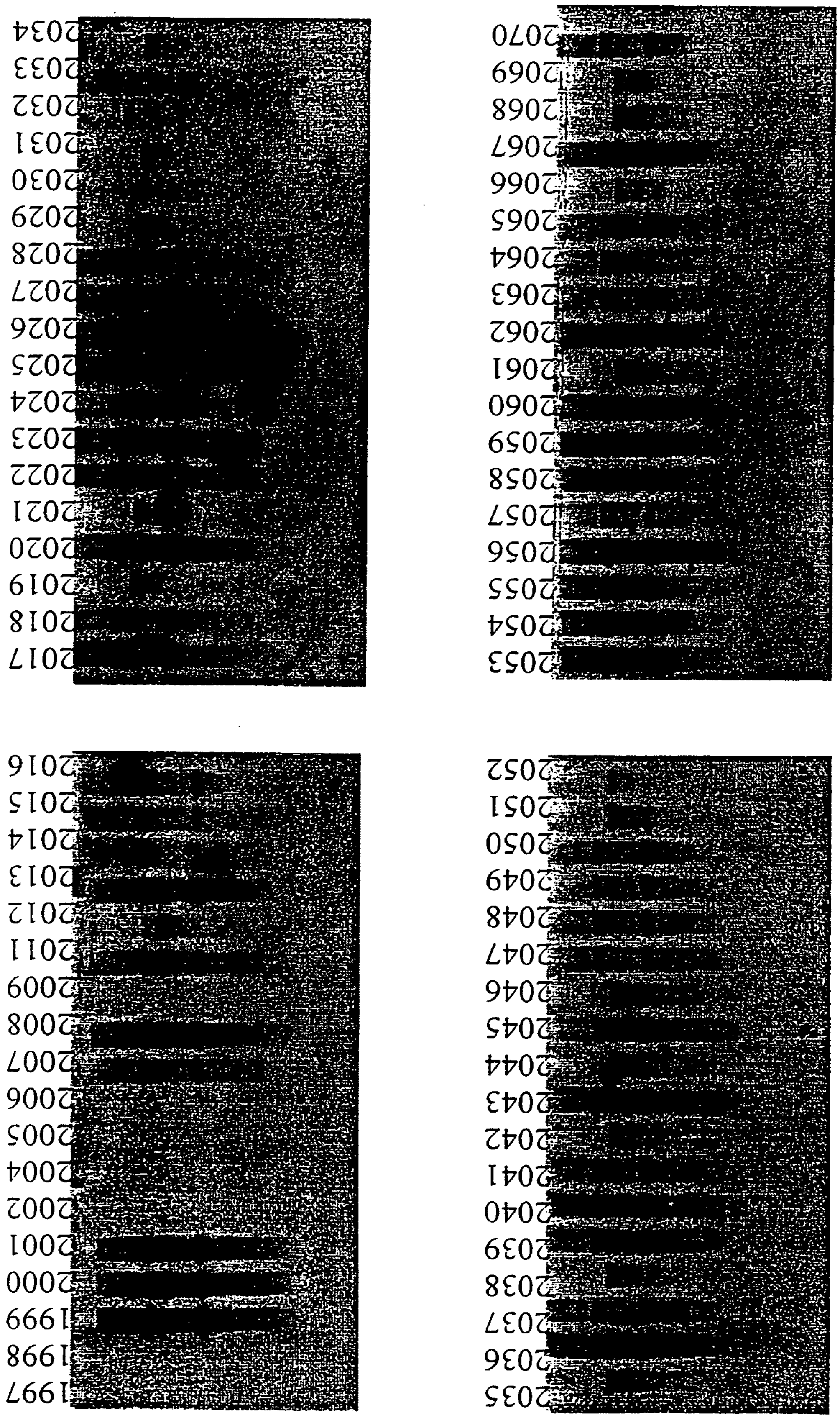

Rhizomania-resistance of T157-01 F1
GUS(-) segregants
Genotype 1
Genotype 2 + 3
Elisa reading
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
1
5
9
13
17
21
25
29
33
37
41
45
49
53
57
61
65
69
73
77
81
85
89
93
97

FIG. 5A

Rhizomania-resistance in T157-01 F1 progeny



Probe: Gus
Enzyme *Sac*I

FIG. 5B

SEQUENCE LISTING

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plants

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<141> 2000-01-26

<150> EP 992002360
<151> 1999-01-27

<160> 4

<170> PatentIn Ver. 2.1

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<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:primer P1

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<213> Artificial Sequence

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<210> 4
<211> 28
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer P4

<400> 4
cgcagatctt taactgctca tcaccaac 28

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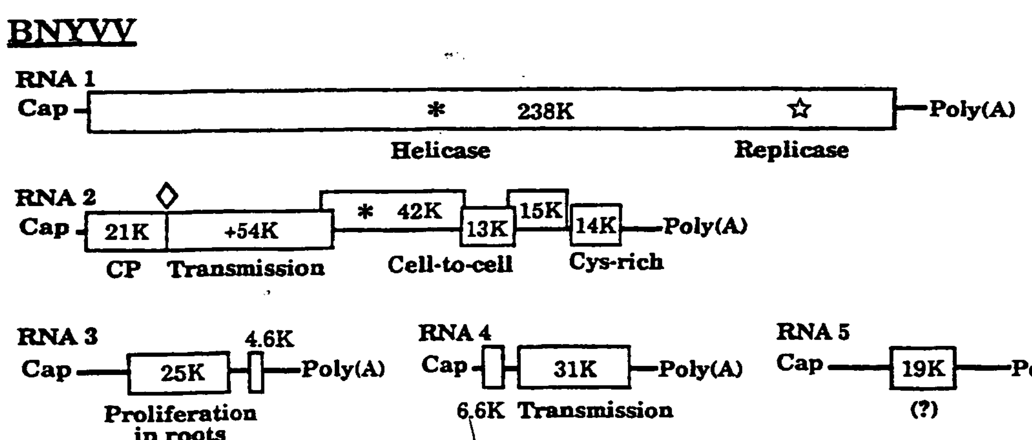
[Continued on next page]

(54) Title: METHOD FOR CONVEYING BNYVV RESISTANCE TO SUGAR BEET PLANTS



(57) Abstract: The present invention relates to a method for conveying resistance to beet necrotic yellow vein virus (BNYVV) to a sugar beet plant, which method comprises the following steps: (a) preparing a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV), (b) introducing said DNA fragment, operatively linked to a promotor that is active in sugar beet plants, into a sugar beet plant cell to obtain a transformed sugar beet cell; and (c) regenerating a transgenic sugar beet plant from the transformed sugar beet plant cell. In addition, the invention relates to a DNA vector harboring a fragment of a least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of BNYVV and transcription and translation regulatory sequences operably linked therewith, as well as to the use of said DNA vector for the transformation of a plant cell. The invention further relates to a plant cell comprising in its genome a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of BNYVV and the use of said plant cell for the regeneration therefrom of a sugar beet plant that is resistant against BNYVV.

WO 00/44915 A1

MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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METHOD FOR CONVEYING BNYVV RESISTANCE TO SUGAR BEET PLANTS

The present invention relates to a method for conveying viral resistance to beet necrotic yellow vein virus (BNYVV) to a sugar beet plant. Furthermore, the invention relates to virus-resistant plants obtained according to this method, as well as to seeds and progeny derived therefrom.

Plant viruses are a serious problem for many of the major agricultural crops. Thus, BNYVV which is transmitted by the soilborne plasmodiophoromycete fungus Polymyxa betae, is the cause of the economically important disease of sugar beet (Beta vulgaris), known as rhizomania. This disease was first reported in Italy in the 1950s and has since become a major threat to sugar beet crops in most growing areas in the world.

Under field conditions BNYVV is generally confined to the subterranean portions of the plant. Infected sugar beet roots exhibit an increased proliferation and necrosis of the lateral roots, a decrease in the total mass of the main root and a consequent reduction in sugar yield. Plant death may also follow infection, particularly if the crop is infected early in season. Occasionally, the virus will ascend into the leaves where it can provide invasive vein-associated chlorotic and necrotic lesions.

BNYVV is a type member of the benyvirus family, which are fungus-transmitted, rod-shaped viruses possessing a divided genome composed of two or more components of single-stranded (ss) RNA. Four different plus sense ssRNA species of BNYVV have been identified, which are referred to as RNAs 1 to 4 in decreasing order of size (RNA 1: 6.8 kb; RNA 2: 4.7 kb; RNA 3: 1.8 kb; RNA 4: 1.5 kb). Some Japanese isolates also contain a fifth RNA component (RNA 5: 1.45 kb). All RNAs have been cloned and sequenced. The genetic map of the viral RNAs is shown in figure 1.

All five RNAs terminate in 3' poly-A tails, and RNAs 1-4 have cap structures at their 5' termini. RNAs 1

and 2 encode basic host-independent "housekeeping" functions, while the smaller RNAs intervene specifically in the natural infection process, including vector-mediated infection of sugar beet roots, proliferation within the root system and production of rhizomania symptoms. Thus, RNA 1 has been shown to code for viral RNA polymerase activity and RNA 2 encodes the 21-kd viral coat-protein. The 3'-proximal half of RNA 2 carries a group of three successive slightly overlapping viral genes, known as the triple gene block (TGB3), that are closely similar to a cluster of three genes in other rod-shaped plant viruses, and are involved in cell to cell movement of the virus. RNA 3 is associated with massive proliferation of fine rootlets in sugar beet and facilitates the spread of the virus in root tissue, whereas RNA 4 increases the efficiency of transmission of the virus by the fungus.

Fungus-transmitted viruses, such as BNYVV may be retained in resting spores in soil for years once a field becomes infested. As there exist no effective chemical or physical methods for eliminating the virus, neither in the plants nor in the soil, the only option for the sugar beet farmer is the use of genetically resistant cultivars. Several companies have provided a number of tolerant, even partially resistant varieties, by transferring wild type tolerance genes into commercially varieties through breeding. This is, however, a very tedious and time-consuming process, generally taking a long time before useful resistant plants are obtained. In addition, under high disease pressure tolerant or partially resistant plants will develop disease symptoms because the resistance level is too low. Another problem with tolerant or only partially resistant plants lies in the fact that virus populations continue to grow resulting in the possible emergence of a BNYVV strain which may break the resistance/tolerance genes.

The rapid revolution in the areas of plant engineering has led to the development of new strategies to confer genetic resistance to viruses. Resistance to viral diseases through the introduction of portions of viral genome sequences has become a new source of resistance. The viral sequence (construct) is transformed into a plant by combining the use of appropriate cell or tissue culture techniques and a DNA delivery system such as the well known Agrobacterium tumefaciens transformation system or the direct DNA transfer mediated by chemicals like polyethylene glycol (PEG).

Known methods of inducing pathogen defense mechanisms in plants are for example described in EP 9687106.0, which relates to a method for inducing resistance to a virus comprising a TGB3 sequence. The publication describes that it was possible to induce BNYVV resistance by a method, which consists of transforming plant cells with a DNA construct corresponding to a fragment of the nucleic acid sequence of the genomic or subgenomic RNA 2 of the BNYVV. The disadvantage of this method is however, that only tolerance is obtained, and not resistance. In tolerant host plants there can still be normal levels of plant virus replication, but the plant shows little or no visible signs of infection, whereas in resistant hosts the virus replication is low or even absent.

In WO 93/25068 viral resistance in plants is induced by plant transformation with a replicase portion (RNA 1) of a plant virus genome. This publication does not refer to sugar beet, nor to BNYVV.

Sugar beet are known to be recalcitrant species in genetic engineering, complicating successful induction of BNYVV resistance. However, because of the extent and impact of the viral disease of BNYVV there is a major interest to improve the sources of genetic resistance in sugar beet plants.

The object of the present invention is, therefore, to provide means to obtain sugar beet plants,

that exhibit resistance to BNYVV. Preferably, they exhibit total resistance or immunity, by combining different approaches and by using methods which do not allow the virus to replicate.

This is achieved by the invention by providing a method for conveying resistance to beet necrotic yellow vein virus (BNYVV) to a sugar beet plant, comprising the following steps:

(a) preparing a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV),

(b) introducing said DNA fragment, operately linked to a promotor that is active in sugar beet plants, into a sugar beet plant cell to obtain a transformed sugar beet cell; and

(c) regenerating a transgenic sugar beet plant from the transformed sugar beet plant cell.

Thus, a sugar beet plant is obtained which exhibits a durable resistance and in which the virus does not replicate. This is a unique aspect of the transformant of the invention and has not been disclosed before.

According to the invention the essential sequence homology of the fragment is a homology of at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

The homology or "degree of similarity" is used to denote nucleotide sequences which when aligned have similar (identical or conservatively replaced) nucleotides in like positions or regions. For example, two nucleotide sequences with at least 85% homology to each other have at least 85% homologous (identical or conservatively replaced nucleotides) in a like position when aligned optimally allowing for up to 3 gaps, with the provison that in respect of the gaps a total of not more than 15 amino acid resides is affected. The degree of similarity may be determined using methods well known

in the art (see, for example, Wilbur, W.J. and Lipman, D.J. "Rapid Similarity Searches of Nucleic Acid and Protein Data Banks." Proceedings of the National Academy of Sciences USA 80, 726-730 (1983) and Myers E.and Miller W. "Optimal Alignments in Linear Space". Comput. Appl. Biosci. 4:11-17(1988)). One programme which may be used in determining the degree of similarity is the MegAlign Lipman-Pearson one pair method (using default parameters) which can be obtained from DNAstar Inc, 1228, Selfpark Street, Madison, Wisconsin, 53715, USA as part of the Lasergene system. The test for homology of the sequence is based on the percent identity which is calculated by Fast DB based on the following parameters: mismatch penalty 1.0, gap penalty (1.00), gap size penalty 0.33 and joining penalty 30.0.

Preferred embodiments of the invention use various fragments having nucleic acid sequences that correspond with the homology indicated or completely to nucleotides 153 to 3258, 169 to 539, 1226 to 1683, 2754 to 3192 or to all of the 6746 nucleotides of RNA 1.

The present invention also includes DNA which hybridises to the DNA of the present invention and which codes for RNA1. Preferably, such hybridisation occurs at, or between, low and high stringency conditions. In general terms, low stringency conditions can be defined as 3 x SCC at about ambient temperature to about 65°C, and high stringency conditions as 0.1 x SSC at about 65°C. SSC is the name of a buffer of 0.15M NaCl, 0.015M trisodium citrate. 3 x SSC is three time as strong as SSC and so on.

The fragment can be introduced into a regenerable plant cell by means of a DNA vector harboring the fragment and transcription and translation regulatory sequences operably linked therewith using standard plant transformation methods such as <u>Agrobacterium</u>-mediated transformation of cells embedded in plant tissues such as cotyledons (Krens et al., Plant Science 116: 97-106; 1996) or polyethylene glycol-mediated DNA uptake of

single cells like the guard cell protoplasts (Hall et al., Nature Biotechnology 14: 1133-1138; 1996). The DNA vector harboring the fragment is also part of the present invention.

The use of constructs constructed such that the gene sequence inhibits or promotes gene expression is quite well understood. A complete gene sequence, under the control of a promoter that operates effectively in the plant, will generally overexpress the gene product, leading to an amplification of the effect of the protein so produced. Sometimes the gene product is reduced: this phenomenon is termed "co-suppression". Downregulating this gene can be done by several techniques. It can be done by 'dominant-negative' constructs. These contain the specific DNA binding domain as well as possible dimerisation domains but are transcriptionally inactive. They 'sit' on the promoters of the target genes and thereby prevent the binding of the endogenous protein. Additionally, reduction of the gene product can also be obtained by using such dominant negative mutation, or by reversing the orientation of the gene sequence with respect to the promoter so that it produces a type of gene product called "antisense" messenger RNA.

A DNA construct according to the invention may be an "antisense" construct generating "antisense" RNA or a "sense" construct (encoding at least part of the functional protein) generating "sense" RNA.

"Antisense RNA" is an RNA sequence which is complementary to a sequence of bases in the corresponding mRNA: complementary in the sense that each base (or the majority of bases) in the antisense sequence (read in the 3' to 5' sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5' to 3' sense. Such antisense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged to generate a transcript with at least part of its sequence complementary to at least part of the coding strand of

the relevant gene (or of a DNA sequence showing substantial homology therewith).

"Sense RNA" is an RNA sequence, which is substantially homologous to at least part of the corresponding mRNA sequence. Such sense RNA may be produced in the cell by transformation with an appropriate DNA construct arranged in the normal orientation so as to generate a transcript with a sequence identical to at least part of the coding strand of the relevant gene (or of a DNA sequence showing substantial homology therewith). Suitable sense constructs may be used to inhibit gene expression (as described in International Patent Publication WO91/08299).

DNA constructs according to the invention may comprise a base sequence at least 10 bases (preferably at least 35 bases) in length for transcription into RNA. There is no theoretical upper limit to the base sequence - it may be as long as the relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences between 100 and 1000 bases in length. The preparation of such constructs is described in more detail below.

As a source of the DNA base sequence for transcription, a suitable cDNA or genomic DNA, RNA or synthetic polynucleotide may be used. The transcriptional initiation region (or promoter) operative in plants may be a constitutive promoter (such as the 35S cauliflower mosaic virus promoter) or an inducible or developmentally regulated promoter, as circumstances require. Suitable DNA sequences for control of expression of the plant expressible genes (including marker genes), such as transcriptional initiation regions, enhancers, leader sequences, non-transcribed leaders and the like, may be derived from any gene that is expressible in a plant cell. Also hybrid promoters combining functional portions of various promoters, or synthetic equivalents thereof can be employed. Apart from constitutive promoters,

inducible promoters, or promoters otherwise regulated in their expression pattern, e.g. developmentally or cell-type specific, may be used to control expression of the expressible genes according to the invention. For example, it may be desirable to modify protein activity at certain stages of the plant's development. Use of a constitutive promoter will tend to affect protein levels and functions in all parts of the plant, while use of a tissue-specific promoter allows more selective control of gene expression and affected functions.

Another option under this invention is to use inducible promoters. Promoters are known which are inducible by pathogens, by stress, by chemicals and by environmental signals. The induction of the gene activity by internal or external induction is within the scope of the present invention. Promoters of this type enable the inducibility of the gene activity in a controlled manner, thus the plant can develop normally without any undue influence by the transgene gene. Promoters that are inducible promoters include those described in DE 4446342 (fungus and auxin inducible PRP-1), WO 96/28561 (fungus inducible PRP-1), EP 0 712 273 (nematode inducible), EP 0 330 479 and US 5,510,474 (stress inducible), WO/96/12814 (cold inducible), and Zeneca's alcohol inducible promoter. Other inducible promoters are described in EP 0 494 724, EP 0 619 844, WO 92/19724. Thus the gene product, whether antisense or sense RNA or the peptide, is only produced in the tissue at the time when its action is required.

As mentioned above, the term "inducible promoter" includes promoters which may be induced chemically. The use of a promoter sequence which is controlled by the application of an external chemical stimulus is most especially preferred. The external chemical stimulus is preferably an agriculturally acceptable chemical, the use of which is compatible with agricultural practice and is not detrimental to plants or mammals. The inducible promoter region most preferably

comprises an inducible switch promoter system such as, for example, a two component system such as the alcA/alcR gene switch promoter system described in the published International Publication No. WO 93/21334, the ecdysone switch system as described in the International Publication No. WO 96/37609 or the GST promoter as described in published International Patent Application Nos. WO 90/08826 and WO 93/031294, the teachings of which are incorporated herein by reference. Such promoter systems are herein referred to as "switch promoters". The switch chemicals used in conjunction with the switch promoters are agriculturally acceptable chemicals making this system particularly useful in the method of the present invention.

The skilled person will be capable of selecting a DNA vector for use in these methods. An example of a suitable vector for the Agrobacterium-mediated transformation is pBIN19. Suitable vectors for the PEG-mediated transformation include the pBluescript vector or pIGPD7 (Hall et al., Nature Biotechnology 14: 1133-1138; 1996). Introduction of the fragment into these vectors can be achieved by means of standard molecular biological techniques as for example described in Sambrook et al., "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory Press; 1989.

Transformed plants obtained by the method of the present invention show absolute resistance, or immunity, to BNYVV. In contrast, previous attempts to convey resistance to plants to BNYVV (Kallerhof et al., Plant Cell Reports 9: 224-228, 1990; and Mannerlof et al., Euphytica 90: 293-299, 1996) or other viruses, such as to tobacco mosaic virus (TMV) (Donson et al., Mol. Plant-Microbe Interact. 6: 635-642; 1993) by transforming plants with portions of the viral genome were less successful. Inoculated leafs still showed symptoms of infection, thus indicating that the resistance is not absolute. It is therefore surprising that the method of

the invention is capable of conveying absolute resistance to BNYVV to sugarbeet plants.

Furthermore the invention relates to a transformed plant cell and a transgenic plant resistant to BNYVV as well as reproducible structures, such as seeds, calluses, buds, embryos, obtained from the transgenic plants and the progeny derived therefrom.

In a preferred embodiment of the invention the resistance described herein can be combined with other types of resistance or tolerance to BNYVV.

The invention will further be illustrated in the following examples and figures, but is not limited thereto.

Figure 1 shows a diagrammatic representation of the genomic organisation of Beet Necrotic Yellow Vein Virus (based on Jupin et al., Seminars in Virology vol 2.2: 112-129; 1991).

Figure 2 shows the physical maps of pVDH239 and pVDH240. LB=left border, RB=right border, P35S=CaMV 35S promoter, NPTII= neomycin phosphotransferase II, T35S=CaMV 35S polyadenylation signal, GUSINT=beta-glucuronidase gene, BNYVVpolTRUNC=BNYVV cDNA1 fragment, Tnos=nopaline synthase gene derived polyadenylation signal. The positions of the main restriction enzyme recognition sites are indicated.

Figure 3 shows a Southern blot analysis by which the number of T-DNA insertions integrated into the genome of the primary sugar beet transformant T157-01 has been determined. The outline of the T-DNA structure of the binary vector pVDH239 is shown at the top.

Figure 4 shows diagrams of the individual ELISA values of the root extracts of sugar beet plants of the populations Cadyx (susceptibel control), Rifle (rhizomania tolerant variety), Rhizor (rhizomania tolerant variety) and T157-01 (GUS-positive F1 individuals) after inoculation with BNYVV-infested soil. Each number at the horizontal axis represents an individual plant.

Figure 5B shows a Southern blot analysis by which the number of T-DNA insertions integrated into the genome of the F1 progeny plants of T157-01 has been determined as well as a diagram of the individual ELISA values of the root extracts of the F1 progeny plants of T157-01) after inoculation with BNYVV-infested soil (figure 5A). The numbers on top of the Southern blots represent the lab-codes of the individual F1 progeny plants. The ELISA values indicated by "Genotype 1" in the lower pannel correspond to the indivduals showing a single band in the Southern blot (2012, 2019, 2021, 2029, 2030, 2031, 2034, 2035, 2038, 2042, 2044, 2046, 2051, 2052, 2061, 2066, 2068, 2069), whereas the ELISA values indicated by "Genotype 2 + 3" in the lower pannel correspond to the individuals showing 2 or 3 bands in the Southern blot (1999, 2000, 2001, 2007, 2008, 2011, 2013, 2014, 2015, 2016, 2017, 2018, 2020, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2032, 2033, 2036, 2037, 2039, 2040, 2041, 2043, 2045, 2047, 2048, 2049, 2050, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2062, 2063, 2064, 2065, 2067, 2070). The ELISA values indicated by "GUS(-) segregants" in the lower pannel correspond to the individual F1 progeny plants which are GUS-negative (1997, 1998, 2002, 2004, 2005, 2006, 2009, rest not shown).

EXAMPLES

EXAMPLE 1

Preparation of contruct with truncated BNYVV replicase sequence

Two primer combinations were used to obtain the cDNA clones of BNYVV for cloning in the tranformation vector (Bouzoubaa et al. J. Gen. Virol. 68:615-626; 1987).

For the 5'-end the primers were

P1: 5'-CGCGGATCCACCATGGCAGATTCGTTC-3' (containing a BamHI and NcoI restriction site and nucleotides identical to nucleotides 153-168), and

P2: 5'-GACGAATTCAAGTCGTCTTTC-3' (EcoRI restriction site and nucleotides complementary to nucleotides 288-301).

For the 3'-end the primers were

P3: 5'-GACGAATTCGAAAGATGAGTCTA-3' (EcoRI site and nucleotides identical to nucleotides 2799-2812), and

P4: 5'-CGCAGATCTTTAACTGCTCATCACCAAC-3' (BglII site and nucleotides complementary to nucleotides 3244-3258 and stop codon).

Before cloning the fragments into the Bluescript vector (Stratagene, La Jolla, CA, USA) the SalI/HincII/AccI site was replaced by a BglII site. After amplification of BNYVV cDNA using P1 and P2 a DNA fragment was obtained which was digested with BamHI and EcoRI and inserted into the modified, BamHI/EcoRI cleaved pBleuscript vector to yield pKSNBPol1. Subsequently, BNYVV cDNA1 was amplified using P3 and P4. The resulting DNA fragment was cleaved by EcoRI and BglII and inserted into pKSBNPol1 cleaved with EcoRI and BglII, yielding pKSNBPpol2.

Thereafter the AccI fragment of BNYVV cDNA1 identical to nucleotides 250-2815 was cloned in AccI cleaved pKSBNPol2. Thus, a pKSBNPoltrunc was obtained which comprised the BNYVV cDNA1 fragment (poltrunc) identical to the nucleotides 153-3258, flanked by a BamHI site at the 5'-end and by a BglII fragment at the 3'-end. The poltrunc fragment was cloned as BamHI-BglII fragment in the BamHI site of pVDH4 such that a functional sense poltrunc fragment was placed behind the CaMV 35S promotor and before the nopaline terminator sequence. The complete construct, carrying the CaMV35S promotor, the poltrunc fragment and nopaline terminator sequence was excised from the plasmid with ClaI and cloned in the binary transformation vector pVDH212 in which the BamHI site was converted into a ClaI site by inserting a molecular linker.

pVDH212 is a pBIN19 derived binary transformation vector, which contains 35S-NPTII (neomycin phosphotransferase under control of the CaMV 35S promotor as a selectable marker gene conferring resistance to kanamycin), as well as 35S-GUSi (gene encoding beta-glucuronidase (Vancanneyt et al., Mol. Gen. Genet. 220: 245-250; 1990). Thus, two different transformation vectors containing the poltrunc construct were obtained: one, pVDH239, with the poltrunc construct in the same transcriptional direction as the NPTII and GUS gene, and another, pVDH240, with the poltrunc construct having an opposite transcriptional direction. The binary transformation vectors pVDH239 and 240 (figure 2) were conjugated and transferred to <u>Agrobacterium tumefaciens</u> LBA4404 (Phabagen Collection, Utrecht, the Netherlands) resulting in HAT1239 (for pVDH239) and HAT1240 (for pVDH240) strains. HAT1239 and HAT1240 were used for transformation of sugar beet.

EXAMPLE 2

<u>Transformation of sugar beet</u>

Methods for <u>Agrobacterium</u>-mediated transformation using binary vectors are well established and known to a person skilled in the art.

To obtain transformed sugar beet plants, O-type B8M5.9 plants were transformed according to Krens et al. (Plant Science 116: 97-106; 1996). Transformation with <u>Agrobacterium</u> HAT1239 yielded 8 transformants: T156-03, T156-06, T156-09, T156-10, T156-13, T156-20, T150-30, and T157-01, and with <u>Agrobacterium</u> HAT1240 12 transformants were obtained: T183-01, T183-02, T183-06, T183-10, T183-16, T183-19, T183-21, T183-23, T183-26, T184-02, T184-03 and T184-04. The transformants were induced to flowering by vernalisation and used to produce seeds either through self-pollination (selfing or S1 seed) or cross-pollination on male sterile plants (hybrid or F1 seed). The pollination was performed in closed pollen chambers

to prevent pollination by sugar beet pollen from a different source.

The F1 seeds were used as starting material to carry out a bioassay for BNYVV resistance. The seedlings were screened initially for GUS activity which indicates the presence of the T-DNA. GUS-negative segregants were used as a negative control in the bioassay. Depending on the total number of T-DNA inserts, the resistance, if present, can segregate within the GUS-positive population. Only T157-01 derived progeny showed BNYVV resistance.

Subsequently, the primary transformant T157-01 was analysed in further detail on Southern blot. Genomic DNA was isolated from leaves, digested seperately with EcoR1, BamHI and SacI and used to prepare a Southern blot which was subsequently probed with GUS. From this experiment it was concluded that the primary transformant contained three T-DNA inserts (figure 3).

EXAMPLE 3

Bioassay

A lateral root bioassay was developed as a single plant test for the selection of rhizomania resistant sugarbeet plants. One-week-old plantlets were transplanted in a standard mixture of 10% rhizomania infected soil and further incubated for 4 weeks. The oonditions for infection were optimised and standardised on the level of the infection pressure and the growth of rootlets (only formation of hairy roots). Incubation conditions were: 18.9-19°C day/night, 70% relative humidity, 10.000 lux white light, pots at 32.5 cm from the lamps.

The amount of virus in the rootlets which was directly correlated with the resistance mechanism in the plant was measured by an ELISA method. The cut-off value for resistant plants was put at an OD-value of 0.2 (Clark et al. J. Virol. Meth. 15:213-222; 1987). Four weeks

after transplantation the lower part of the roots was used for ELISA. The root piece was dried on a filter paper and transferred to a mortar. Extraction buffer was added at a ratio of 10 volume equivalents of the root weight (dilution 1/10). Root juice was extracted by hand crushing. The juice was transferred to 1.5 ml tubes and centrifuged (300 rpm, 10 minutes) the supernatants were kep on ice and assayed. Populations tested were:

Population	Number of plants	Remarks
T 157-01 (F1)	73	GUS(+) individuals
Cadyx F 052	26	sensitive control
Rhizor F 202	27	tolerant control
Rifle	28	tolerant control

Within the group of T157-01 GUS(+) plants two categories can be observed (figure 4). One category displays immunity to BNYVV (ELISA cut-off 0.2) with an average ELISA value of 0.006 which is the background level of the experimental system. The other category shows normal susceptibility with an average ELISA value in the range of the susceptible control.

The infection pressure in this bioassay was high due to the increase of temperature during a part of the infection period. Consequently there was no clear distinction between Cadyx and Rizor/Rifle (figure 4). On the other hand, the resistant plants selected in the T 157-01 F1 progeny could be considered as totally resistant independent of the rhizomania infection pressure (figure 4). In conclusion, the introduced construct containing the BNYVV cDNA1 fragment resulted in a dramatic negative effect on the multiplication of BNYVV in the lateral roots of the inoculated transgenic sugar beet plants which effectively rendered the plants completely resistant to rhizomania.

In order to explain the segregation of the GUS(+) population in a resistant and a suceptible category the individual plants were analysed in a separate experiment for resistance to BNYVV and simultaneously for the presence of the T-DNAs by Southern analysis using SacI as restriction enzyme and GUS as a probe. The results, as depicted in Figure 5A and 5B, point out that the GUS(+) plants within this population which contained a single band on the Southern blot were all susceptible (average ELISA value 0.63), whereas the GUS(+) plants which contained either 2 or 3 bands on the Southern blot were all resistant (average ELISA value: 0.21). The GUS(-) segregants were all susceptible (average ELISA value 0.80). Apparently the resistant phenotype is linked to the presence of the upper and lower band of this particular band pattern obtained on Southern blot, whereas the presence of the middle band is not linked to the resistant phenotype. It is therefore concluded that the segregation of the GUS(+) population into a suseptible and a resistant category can be explained by the fact that one of the T-DNAs which can result in a GUS(+) phenotype does not contribute to the resistance to BNYVV.

CLAIMS

1. Method for conveying resistance to beet necrotic yellow vein virus (BNYVV) to a sugar beet plant, comprising the following steps:

(a) preparing a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV),

(b) introducing said DNA fragment, operately linked to a promotor that is active in sugar beet plants, into a sugar beet plant cell to obtain a transformed sugar beet cell; and

(c) regenerating a transgenic sugar beet plant from the transformed sugar beet plant cell.

2. Method as claimed in claim 1, wherein the essential sequence homology of the fragment is a homology of at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

3. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 153 to 3258 of RNA 1 of said virus.

4. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 169 to 539 of RNA 1 of said virus.

5. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 1226 to 1683 of RNA 1 of said virus.

6. Method according to claim 1 or 2 characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in

claims 1 and 2 to nucleotides 2754 to 3192 of RNA 1 of said virus.

7. Method according to claim 1 or 2 characterized in that the fragment consists of 6746 nucleotides.

8. Method as claimed in claims 1-7 characterized in that the fragment is introduced into the cell by means of a DNA vector harboring the fragment and transcription and translation regulatory sequences operably linked therewith.

9. DNA vector harboring a fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV), and transcription and translation regulatory sequences operably linked therewith.

10. DNA vector as claimed in claim 9, wherein the essential sequence homology of the fragment is a homology of at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

11. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 153 to 3258 of RNA 1 of said virus.

12. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 169 to 539 of RNA 1 of said virus.

13. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 1226 to 1683 of RNA 1 of said virus.

14. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in

claims 9 and 10 to nucleotides 2754 to 3192 of RNA 1 of said virus.

15. DNA vector according to claim 9 or 10, characterized in that the fragment consists of 6746 nucleotides.

16. Use of a DNA vector as claimed in claims 9-15 for the transformation of a plant cell.

17. Plant cell comprising in its genome a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV).

18. Plant cell as claimed in claim 17, wherein the essential sequence homology of the fragment is a homology of at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

19. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 153 to 3258 of RNA 1 of said virus.

20. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 169 to 539 of RNA 1 of said virus.

21. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 1226 to 1683 of RNA 1 of said virus.

22. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 2754 to 3192 of RNA 1 of said virus.

23. Plant cell according to claim 17 or 18, characterized in that the fragment consists of 6746 nucleotides.

24. Plant cell as claimed in claims 17-23 being part of a sugar beet plant that is resistant against BNYVV.

25. Use of a plant cell as claimed in claims 17-23 for the regeneration therefrom of a sugar beet plant that is resistant against BNYVV.

26. Sugar beet plant consisting at least partly of plant cells as claimed in claims 17-23.

27. Progeny of a sugar beet plant as claimed in claim 26.

28. Seeds of a sugar beet plant as claimed in claim 26.

29. Vegetatively reproducible structures, such as calluses, buds, embryos, from a plant according to claim 26 or progeny according to claim 27.

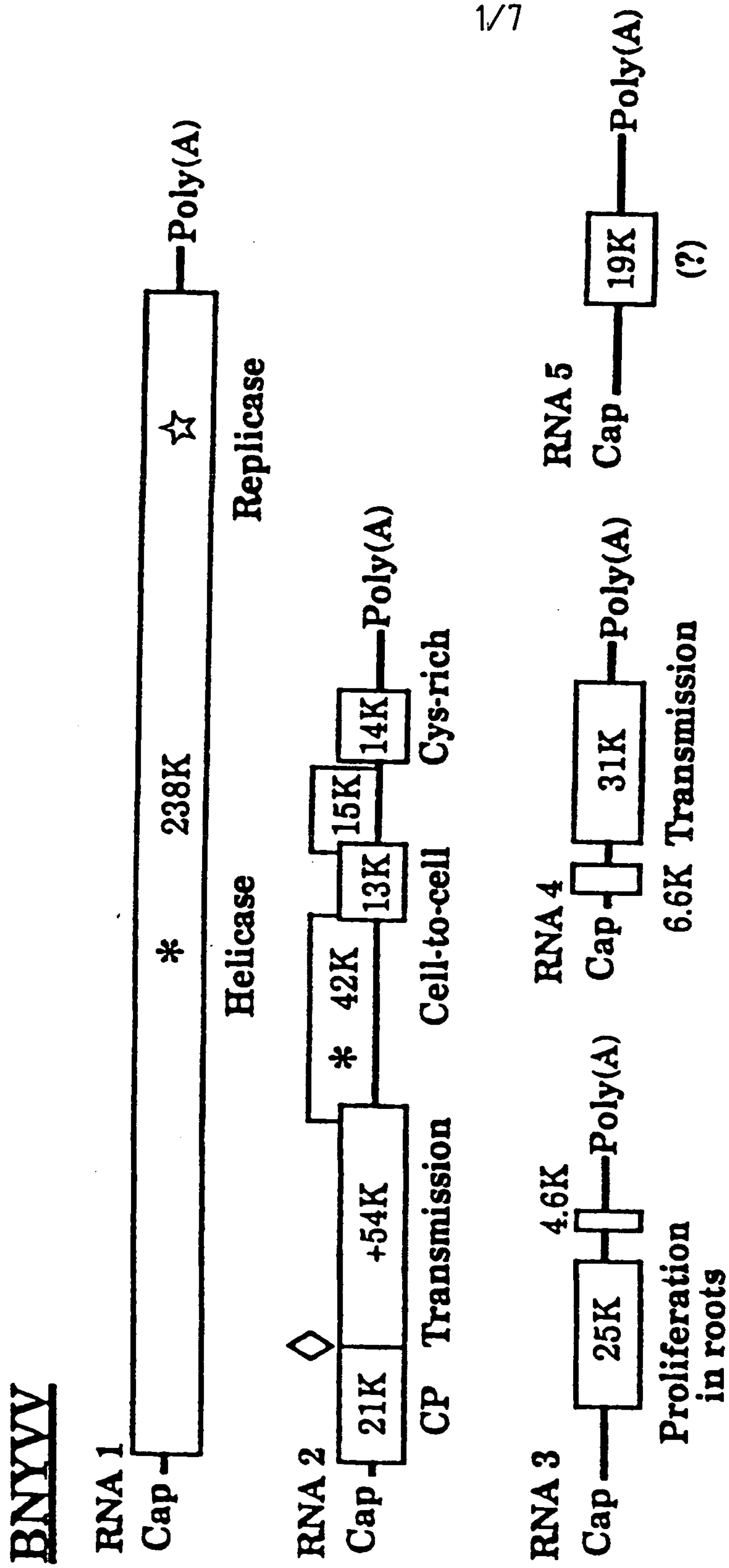
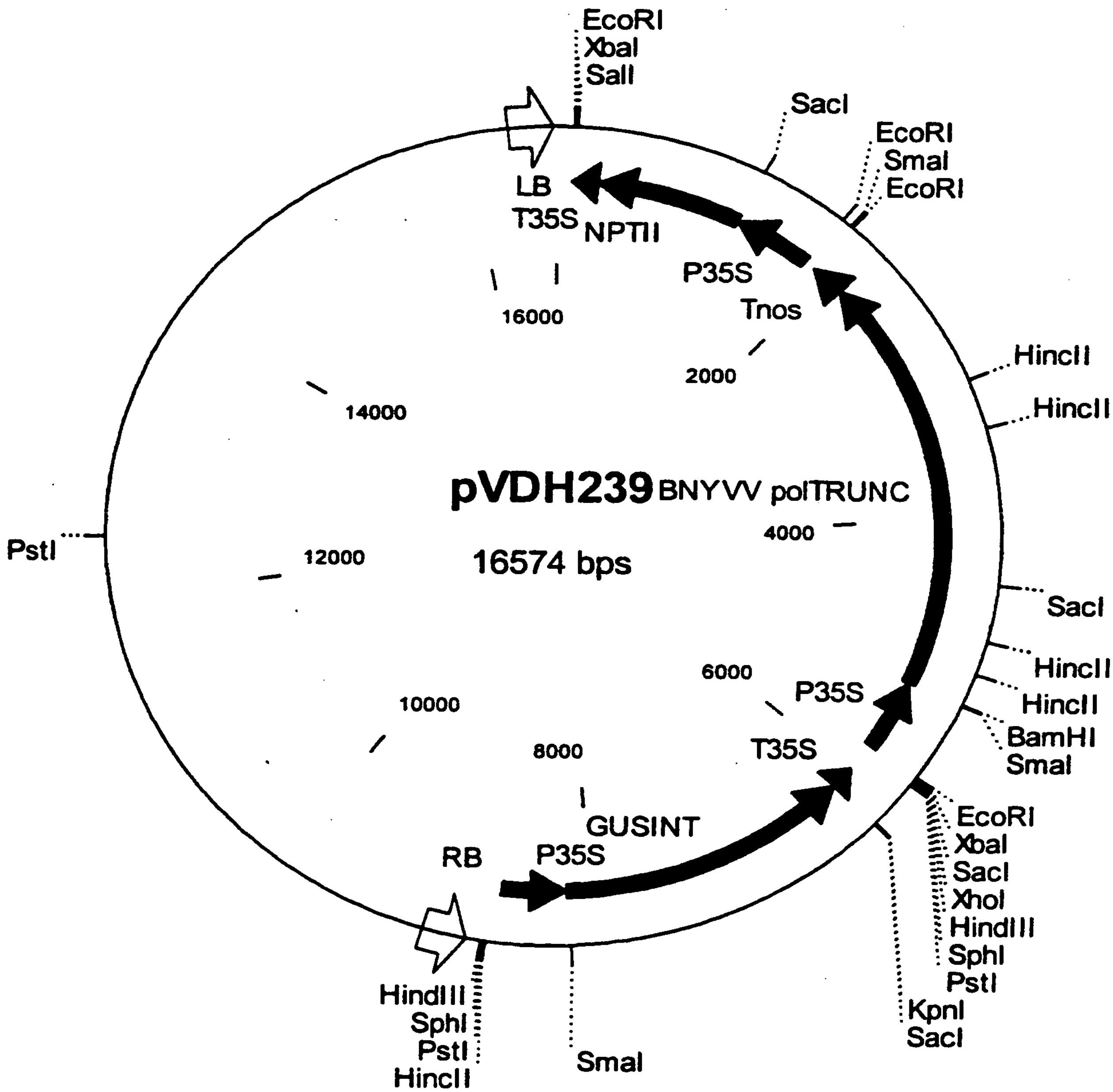

BNYVV
RNA 1
Cap
238K
Poly(A)
Helicase
Replicase
RNA 2
Cap
21K
+54K
42K
13K
15K
14K
Poly(A)
CP
Transmission
Cell-to-cell
Cys-rich
RNA 3
Cap
25K
4.6K
Poly(A)
Proliferation
in roots
RNA 4
Cap
31K
Poly(A)
6.6K
Transmission
RNA 5
Cap
19K
Poly(A)
(?)

FIG. 1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HincII
HincII
14000
pVDH239 BNYVV polTRUNC
16574 bps
4000
PstI
12000
SacI
6000
HincII
P35S
HincII
10000
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GUSINT
SacI
RB
P35S
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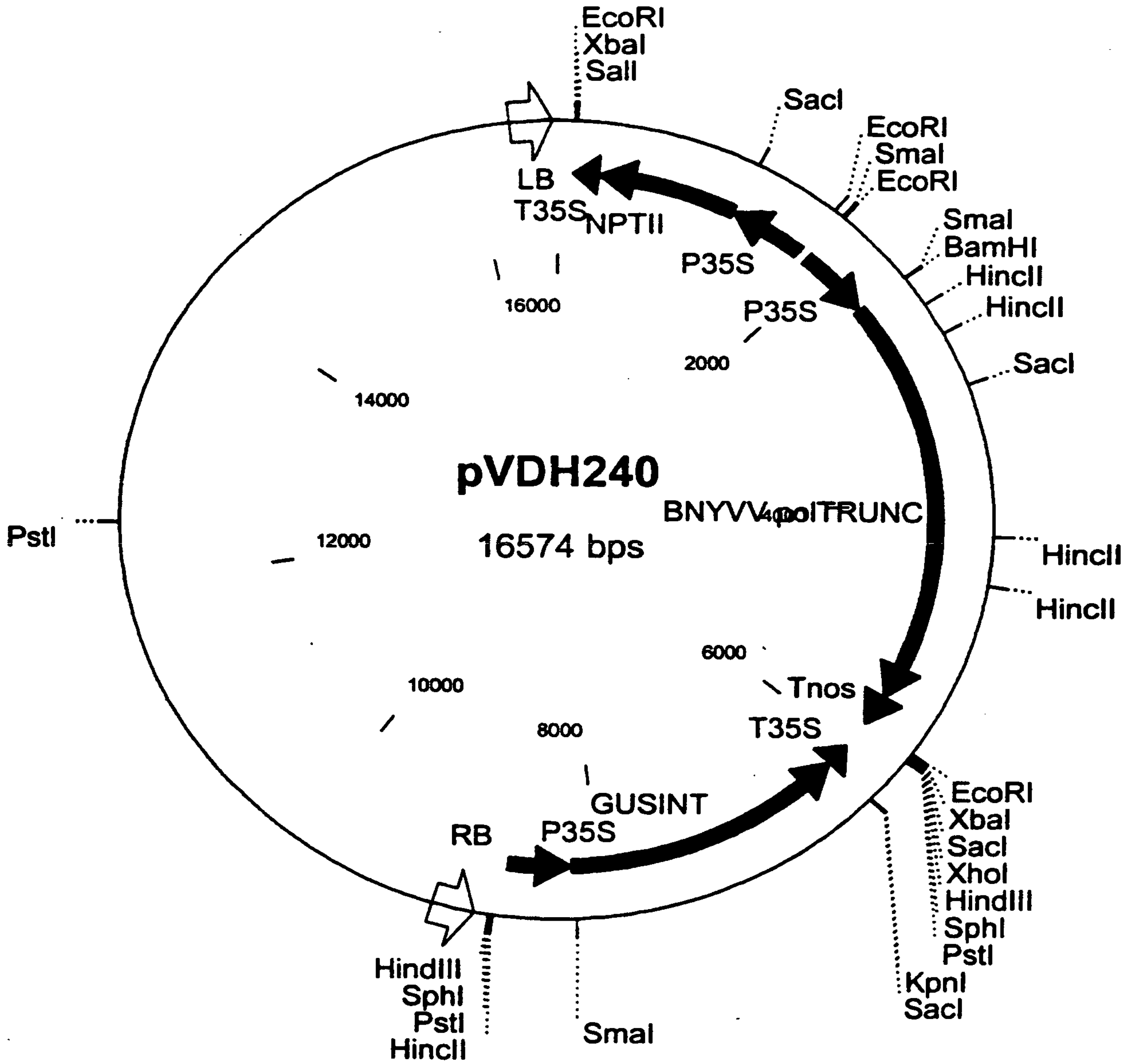
FIG. 2A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NPTII
P35S
P35S
16000
2000
14000
pVDH240
BNYVVpolTRUNC
16574 bps
4000
PstI
12000
HincII
HincII
6000
Tnos
10000
T35S
8000
GUSINT
RB
P35S
EcoRI
XbaI
SacI
XhoI
HindIII
SphI
PstI
KpnI
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HindIII
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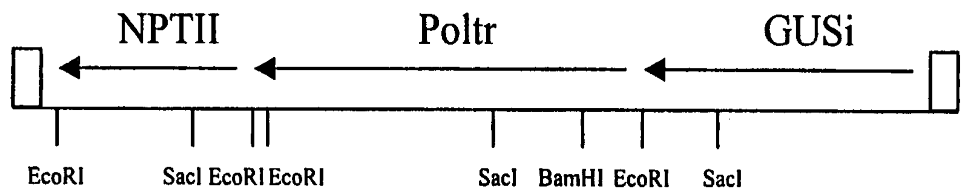
PstI
HincII
SmaI

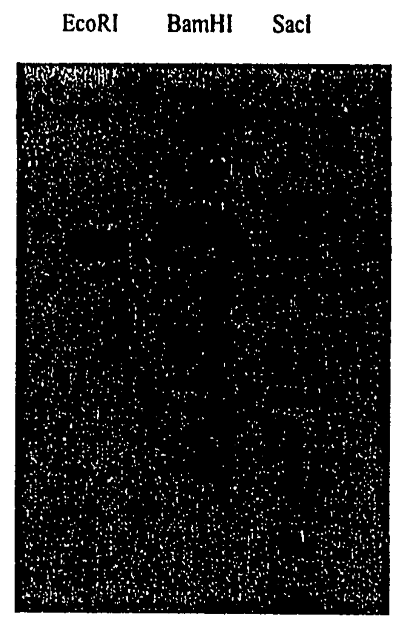
FIG. 2B

Southern analysis sugar beet transformant T157-01 (pVDH239)

T-DNA:



Probe: GUSi

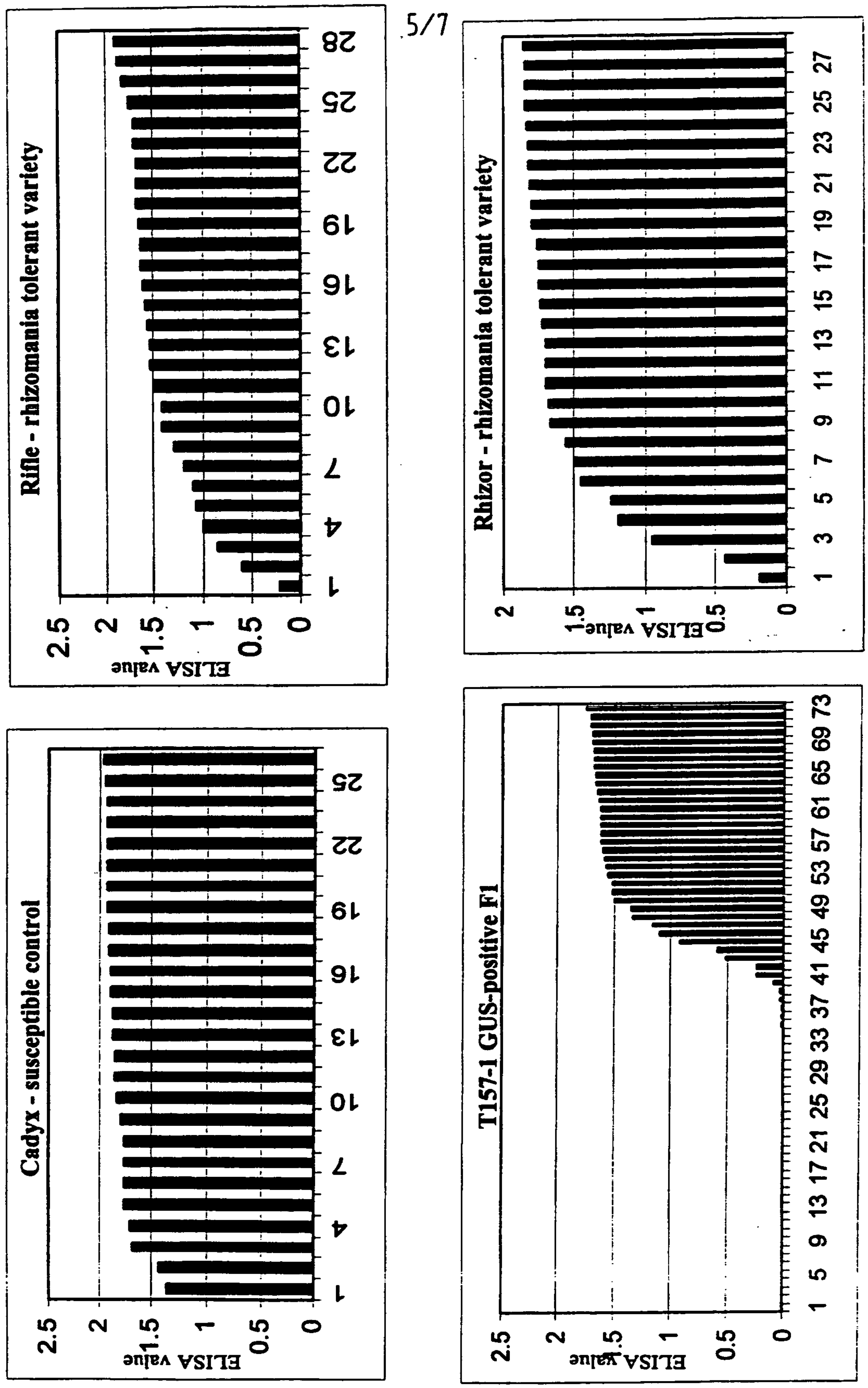


Conclusion: T157-01 contains 3 inserts

FIG. 3

FIG.4

Bioassay rhizomania resistance T157-1



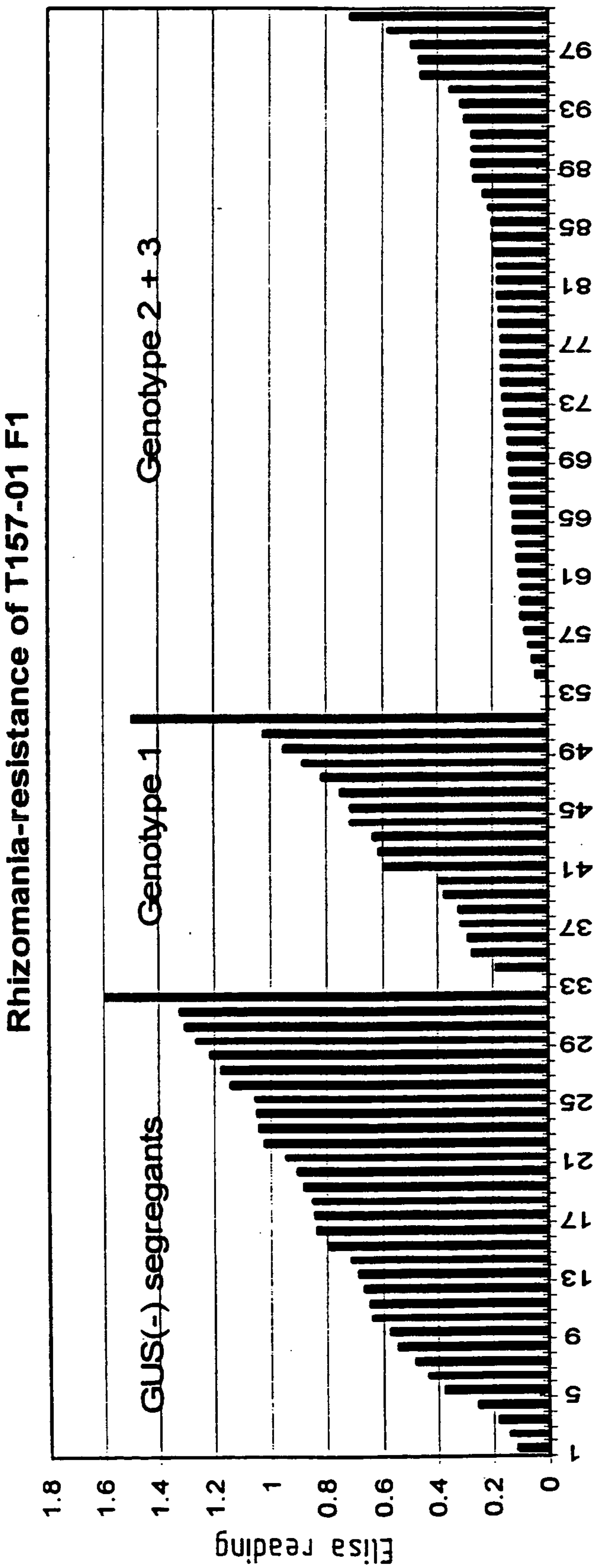
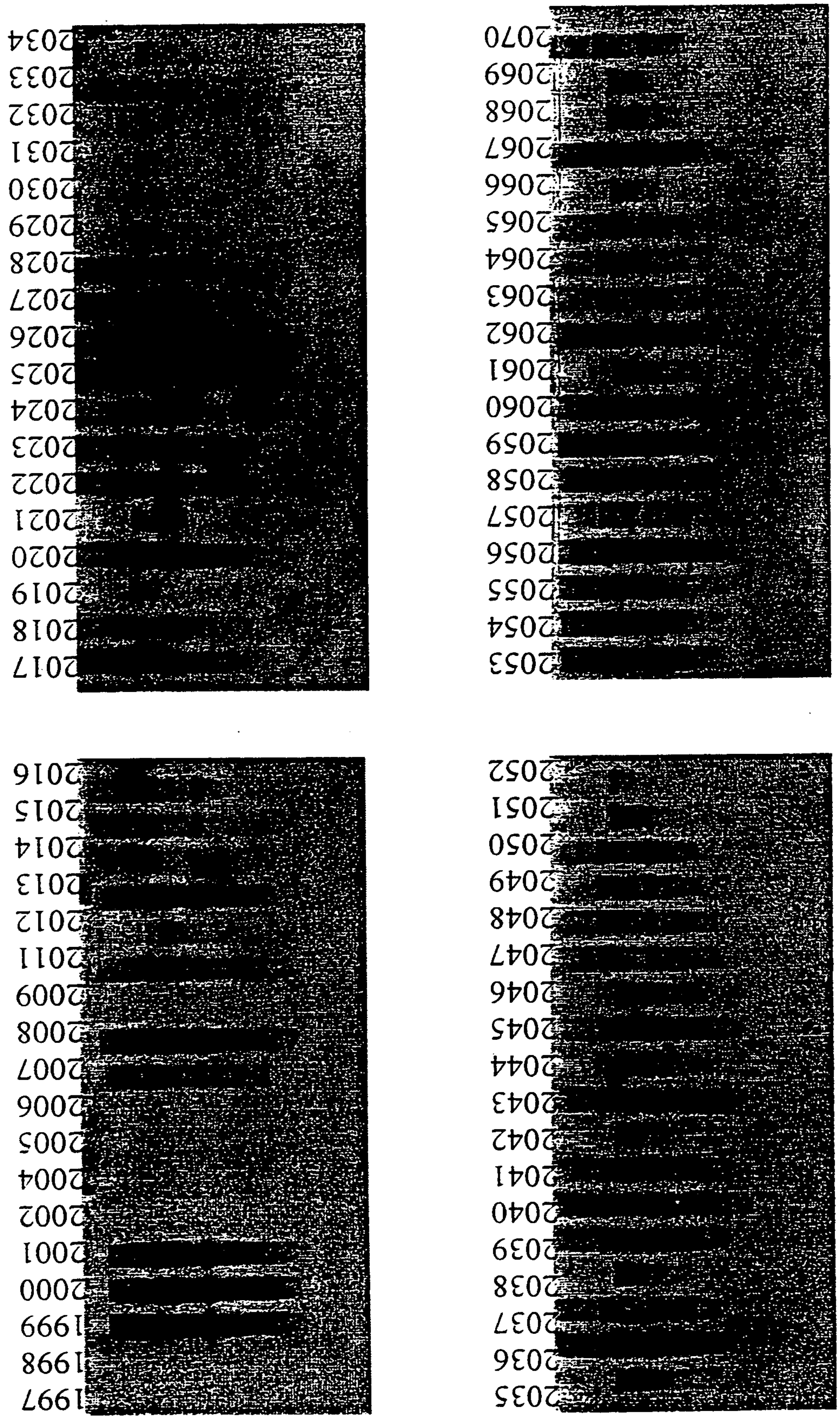

Rhizomania-resistance of T157-01 F1
GUS(-) segregants
Genotype 1
Genotype 2 + 3
Elisa reading
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
5
9
13
17
21
25
29
33
37
41
45
49
53
57
61
65
69
73
77
81
85
89
93
97

FIG. 5A

Rhizomania-resistance in T157-01 F1 progeny



Probe: Gus
Enzyme *Sac*I

FIG. 5B

SEQUENCE LISTING

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<150> EP 992002360
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/00609

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N15/40 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BOUZOUBAA S ET AL: "NUCLEOTIDE SEQUENCE OF BEET NECROTIC YELLOW VEIN VIRUS RNA-1" JOURNAL OF GENERAL VIROLOGY, vol. 68, 1 January 1987 (1987-01-01), pages 615-626, XP000650387 ISSN: 0022-1317 cited in the application the whole document	1-29
A	WO 98 07875 A (SES EUROP N V S A ;LEFEBVRE MARC (BE); WEYENS GUY (BE); BLEYKASTEN) 26 February 1998 (1998-02-26) abstract, page 5; page 14, line 22-24	1-29
A	WO 91 13159 A (BIOSEM) 5 September 1991 (1991-09-05) abstract, page 13,18,19	1-29

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 June 2000	14/06/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Holtorf, S

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MANNERLOF, M., ET AL.: "reduced titer of BNYVV in transgenic sugar beets expressing the BNYVV coat protein" EUPHYTICA, vol. 90, no. 3, 1996, pages 293-299, XP002112359 cited in the application the whole document	1-29
A	SAITO,M., ET AL.: "complete nucleotide sequence of the Japanese isolate S of beet necrotic yellow vein virus RNA and comparison with European isolates" ARCHIVES OF VIROLOGY, vol. 141, 1996, pages 2163-2175, XP002112360 the whole document	1-29
A	HEHN, A., ET AL.: "evidence for in vitro and in vivo autocatalytic processing of the primary translation product of beet necrotic yellow vein virus RNA 1 by a papain-like proteinase" ARCHIVES OF VIROLOGY, vol. 142, 1997, pages 1051-1058, XP002112363 the whole document	1-29

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/00609

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9807875 A	26-02-1998	AU 3935097 A	06-03-1998
		EP 0938574 A	01-09-1999
		PL 331837 A	02-08-1999
WO 9113159 A	05-09-1991	FR 2658987 A	06-09-1991
		FR 2658988 A	06-09-1991
		AT 129745 T	15-11-1995
		DE 69114275 D	07-12-1995
		DE 69114275 T	13-06-1996
		DK 517833 T	04-12-1995
		EP 0517833 A	16-12-1992
		ES 2079647 T	16-01-1996

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference 4/WR87/cm/4	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP00/00609	International filing date *(day/month/year)* 26/01/2000	Priority date *(day/month/year)* 27/01/1999
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant SES EUROPE N.V./S.A. et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand 21/08/2000	Date of completion of this report 03.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Claes, B Telephone No. +49 89 2399 8429

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/00609

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-16 as originally filed

Claims, No.:

1-29 as received on 02/04/2001 with letter of 02/04/2001

Drawings, sheets:

1/7-7/7 as originally filed

Sequence listing part of the description, pages:

1-2, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-26
	No:	Claims	27-29
Inventive step (IS)	Yes:	Claims	1-26
	No:	Claims	27-29
Industrial applicability (IA)	Yes:	Claims	1-29
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I

Basis of the opinion

The application contains a sequence listing of 2 pages.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The following documents are referred to:

   D1 = WO98/07875

   D2 = WO91/13159

   D3 = Bouzoubaa et al. (1987), J. Gen. Virol., 68, 615-626.

2. The application deals with an alternative method for conveying resistance in beet against BNYVV virus. Earlier approaches had used the Triple Gene Block (TBG3) related genes to engineer resistance (D1) or nucleotide fragments that encode parts of the capsid of the virus (D2). RNA-1 of BNYVV was thus far not used for generating resistance in beet, its sequence had been known though from D3.

3. The prior art cited in the International Search Report neither diploses nor suggests the subject matter of claims 1-26. Consequently, novelty and inventive step can be acknowledged for the subject matter of claims 1-26.

4. In view of the fact that claim 26 merely defines at least partly transformed plants, the progeny, the seeds and vegetatively reproducible structures of claims 27-29 do not necessarily comprise any transgene. Consequently, the subject matter of these claims also embraces known plant material (Article 33(2) PCT).

Re Item VIII

Certain observations on the international application

1. The application does not exactly define to what sequence of DNA 1 the claim refer to. Consequently, such references throughout the claims are not conform the

clarity requirement of Article 6 PCT.

CLAIMS

1. Method for conveying resistance to beet necrotic yellow vein virus (BNYVV) to a sugar beet plant, comprising the following steps:

(a) preparing a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the <u>b</u>eet <u>n</u>ecrotic <u>y</u>ellow <u>v</u>ein <u>v</u>irus (BNYVV),

(b) introducing said DNA fragment, operately linked to a promotor that is active in sugar beet plants, into a sugar beet plant cell to obtain a transformed sugar beet cell; and

(c) regenerating a transgenic sugar beet plant from the transformed sugar beet plant cell.

2. Method as claimed in claim 1, wherein the essential sequence homology of the fragment is a homology of at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

3. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 153 to 3258 of RNA 1 of said virus.

4. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 169 to 539 of RNA 1 of said virus.

5. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 1226 to 1683 of RNA 1 of said virus.

6. Method according to claim 1 or 2 characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in

claims 1 and 2 to nucleotides 2754 to 3192 of RNA 1 of said virus.

7. Method according to claim 1 or 2 characterized in that the fragment consists of 6746 nucleotides.

8. Method as claimed in claims 1-7 characterized in that the fragment is introduced into the cell by means of a DNA vector harboring the fragment and transcription and translation regulatory sequences operably linked therewith.

9. DNA vector harboring a fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the <u>b</u>eet <u>n</u>ecrotic <u>y</u>ellow <u>v</u>ein <u>v</u>irus (BNYVV), and transcription and translation regulatory sequences operably linked therewith.

10. DNA vector as claimed in claim 9, wherein the essential sequence homology of the fragment is a homology of at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

11. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 153 to 3258 of RNA 1 of said virus.

12. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 169 to 539 of RNA 1 of said virus.

13. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 1226 to 1683 of RNA 1 of said virus.

14. DNA vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in

claims 9 and 10 to nucleotides 2754 to 3192 of RNA 1 of said virus.

15. DNA vector according to claim 9 or 10, characterized in that the fragment consists of 6746 nucleotides.

16. Use of a DNA vector as claimed in claims 9-15 for the transformation of a plant cell.

17. Plant cell comprising in its genome a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV).

18. Plant cell as claimed in claim 17, wherein the essential sequence homology of the fragment is a homology of at least 70%, preferably at least 80%, more preferably at least 90%, most preferably at least 95%.

19. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 153 to 3258 of RNA 1 of said virus.

20. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 169 to 539 of RNA 1 of said virus.

21. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 1226 to 1683 of RNA 1 of said virus.

22. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 2754 to 3192 of RNA 1 of said virus.

23. Plant cell according to claim 17 or 18, characterized in that the fragment consists of 6746 nucleotides.

24. Plant cell as claimed in claims 17-23 being part of a sugar beet plant that is resistant against BNYVV.

25. Use of a plant cell as claimed in claims 17-23 for the regeneration therefrom of a sugar beet plant that is resistant against BNYVV.

26. Sugar beet plant consisting at least partly of plant cells as claimed in claims 17-23.

27. Progeny of a sugar beet plant as claimed in claim 26.

28. Seeds of a sugar beet plant as claimed in claim 26.

29. Vegetatively reproducible structures, such as calluses, buds, embryos, from a plant according to claim 26 or progeny according to claim 27.

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/82, 15/40, 5/10, A01H 5/00 — A1

(11) International Publication Number: WO 00/44915

(43) International Publication Date: 3 August 2000 (03.08.00)

(21) International Application Number: PCT/EP00/00609

(22) International Filing Date: 26 January 2000 (26.01.00)

(30) Priority Data:
99200236.0 27 January 1999 (27.01.99) EP

(71) Applicant *(for all designated States except US)*: SES EUROPE N.V./S.A. [BE/BE]; Industriepark, Soldatenplein Z 2, No. 15, B-3300 Tienen (BE).

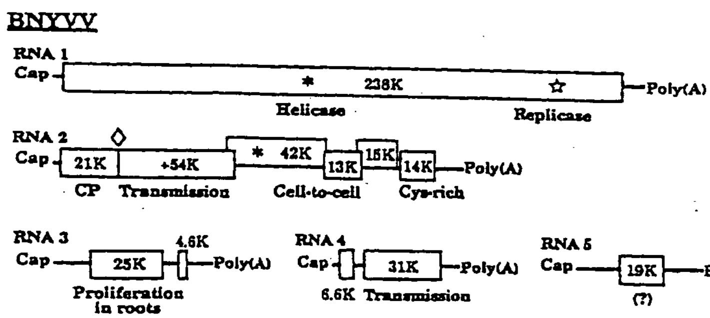
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: RICHARDS, Kenneth [FR/FR]; 2A, rue Principle, F-67370 Pfulgriesheim (FR). JONARD, Gérard [FR/FR]; 9, quai Chanione Winterer, F-67000 Strasbourg (FR). GUILLEY, Hubert [FR/FR]; 32, rue Herbe, F-67370 Berstett (FR). VAN DUN, Cornelis, Maria, Petrus [NL/NL]; 40, Faunaberg, 4708 CC Roosendaal (NL).

(74) Agent: VAN SOMEREN, Petronella, Francisca, Hendrika, Maria; Arnold & Siedsma, Sweelinckplein 1, NL-2517 GK The Hague (NL).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR CONVEYING BNYVV RESISTANCE TO SUGAR BEET PLANTS



(57) Abstract

The present invention relates to a method for conveying resistance to beet necrotic yellow vein virus (BNYVV) to a sugar beet plant, which method comprises the following steps: (a) preparing a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV), (b) introducing said DNA fragment, operatively linked to a promotor that is active in sugar beet plants, into a sugar beet plant cell to obtain a transformed sugar beet cell; and (c) regenerating a transgenic sugar beet plant from the transformed sugar beet plant cell. In addition, the invention relates to a DNA vector harboring a fragment of a least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of BNYVV and transcription and translation regulatory sequences operably linked therewith, as well as to the use of said DNA vector for the transformation of a plant cell. The invention further relates to a plant cell comprising in its genome a DNA fragment of at least 15 nucleotides in a sequence that is essentially homologous to the corresponding nucleotide sequence of the genomic RNA 1 of BNYVV and the use of said plant cell for the regeneration therefrom of a sugar beet plant that is resistant against BNYVV.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 4/WR87/4	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/EP 00/00609	International filing date *(day/month/year)* 26/01/2000	(Earliest) Priority Date *(day/month/year)* 27/01/1999
Applicant SES EUROPE N.V./S.A. et al		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

      [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

      [X] contained in the international application in written form.
      [X] filed together with the international application in computer readable form.
      [ ] furnished subsequently to this Authority in written form.
      [ ] furnished subsequently to this Authority in computer readble form.
      [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
      [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title**,

   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

   [X] the text is approved as submitted by the applicant.
   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [X] as suggested by the applicant.
   [ ] because the applicant failed to suggest a figure.
   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

Form PCT/ISA/210 (first sheet) (July 1998)

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PATENT COOPERATION TREATY

REC'D 07 MAY 2001
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference 4/WR87/cm/4	**FOR FURTHER ACTION** See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/EP00/00609	International filing date *(day/month/year)* 26/01/2000	Priority date *(day/month/year)* 27/01/1999
International Patent Classification (IPC) or national classification and IPC C12N15/82		
Applicant SES EUROPE N.V./S.A. et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☐ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21/08/2000	03.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Claes, B Telephone No. +49 89 2399 8429

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

07 MAY 2001
WIPO PCT

International application No. PCT/EP00/00609

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-16 as originally filed

Claims, No.:

1-29 as received on 02/04/2001 with letter of 02/04/2001

Drawings, sheets:

1/7-7/7 as originally filed

Sequence listing part of the description, pages:

1-2, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-26
	No:	Claims	27-29
Inventive step (IS)	Yes:	Claims	1-26
	No:	Claims	27-29
Industrial applicability (IA)	Yes:	Claims	1-29
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I

Basis of the opinion

The application contains a sequence listing of 2 pages.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The following documents are referred to:

   D1 = WO98/07875
   D2 = WO91/13159
   D3 = Bouzoubaa et al. (1987), J. Gen. Virol., 68, 615-626.

2. The application deals with an alternative method for conveying resistance in beet against BNYVV virus. Earlier approaches had used the Triple Gene Block (TBG3) related genes to engineer resistance (D1) or nucleotide fragments that encode parts of the capsid of the virus (D2). RNA-1 of BNYVV was thus far not used for generating resistance in beet, its sequence had been known though from D3.

3. The prior art cited in the International Search Report neither diploses nor suggests the subject matter of claims 1-26. Consequently, novelty and inventive step can be acknowledged for the subject matter of claims 1-26.

4. In view of the fact that claim 26 merely defines at least partly transformed plants, the progeny, the seeds and vegetatively reproducible structures of claims 27-29 do not necessarily comprise any transgene. Consequently, the subject matter of these claims also embraces known plant material (Article 33(2) PCT).

Re Item VIII

Certain observations on the international application

1. The application does not exactly define to what sequence of DNA 1 the claim refer to. Consequently, such references throughout the claims are not conform the

clarity requirement of Article 6 PCT.

CLAIMS

(38)

1. Method for conveying resistance to beet necrotic yellow vein virus (BNYVV) to a sugar beet plant, comprising the following steps:

(a) preparing a DNA fragment of at least 15 nucleotides in a sequence that is at least 70% homologous to the corresponding nucleotide sequence of the genomic RNA 1 of the beet necrotic yellow vein virus (BNYVV),

(b) introducing said DNA fragment, operately linked to a promotor that is active in sugar beet plants, into a sugar beet plant cell to obtain a transformed sugar beet cell; and

(c) regenerating a transgenic sugar beet plant from the transformed sugar beet plant cell.

2. Method as claimed in claim 1, wherein the DNA fragment is at least 80%, preferably at least 90%, more preferably at least 95% homologous to the corresponding nucleotide sequence of the genomic RNA 1 of said virus.

3. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 153 to 3258 of RNA 1 of said virus.

4. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 169 to 539 of RNA 1 of said virus.

5. Method according to claim 1 or 2, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 1 and 2 to nucleotides 1226 to 1683 of RNA 1 of said virus.

6. Method according to claim 1 or 2 characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in

claims 1 and 2 to nucleotides 2754 to 3192 of RNA 1 of said virus.

7. Method according to claim 1 or 2 characterized in that the fragment consists of 6746 nucleotides.

8. Method as claimed in claims 1-7 characterized in that the fragment is introduced into the cell by means of a DNA vector harboring the fragment and transcription and translation regulatory sequences operably linked therewith.

9. Transformation vector for conveying resistance to BNYVV to a plant, harboring a fragment of at least 15 nucleotides in a sequence that is at least 70% homologous to the corresponding nucleotide sequence of the genomic RNA 1 of said virus, and transcription and translation regulatory sequences operably linked there-with.

10. Vector as claimed in claim 9, wherein the fragment is at least 80%, preferably at least 90%, more preferably at least 95% homologous to the corresponding nucleotide sequence of the genomic RNA 1 of said virus.

11. Vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 153 to 3258 of RNA 1 of said virus.

12. Vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 169 to 539 of RNA 1 of said virus.

13. Vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 1226 to 1683 of RNA 1 of said virus.

14. Vector according to claim 9 or 10, characterized in that the fragment has a nucleic acid

sequence that corresponds with the homology indicated in claims 9 and 10 to nucleotides 2754 to 3192 of RNA 1 of said virus.

15. Vector according to claim 9 or 10, characterized in that the fragment consists of 6746 nucleotides.

16. Use of a vector as claimed in claims 9-15 for the transformation of a plant cell.

17. Plant cell, exibiting a resistance to BNYVV, comprising in its genome a DNA fragment of at least 15 nucleotides in a sequence which is at least 70% homologous to the corresponding nucleotide sequence of the genomic RNA 1 of said virus.

18. Plant cell as claimed in claim 17, wherein the fragment is at least 80%, preferably at least 90%, more preferably at least 95% homologous to the corresponding nucleotide sequence of the genomic RNA 1 of said virus.

19. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 153 to 3258 of RNA 1 of said virus.

20. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 169 to 539 of RNA 1 of said virus.

21. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 1226 to 1683 of RNA 1 of said virus.

22. Plant cell according to claim 17 or 18, characterized in that the fragment has a nucleic acid sequence that corresponds with the homology indicated in claims 17 and 18 to nucleotides 2754 to 3192 of RNA 1 of said virus.

23. Plant cell according to claim 17 or 18, characterized in that the fragment consists of 6746 nucleotides.

24. Plant cell as claimed in claims 17-23 being part of a sugar beet plant that is resistant against BNYVV.

25. Use of a plant cell as claimed in claims 17-23 for the regeneration therefrom of a sugar beet plant that is resistant against BNYVV.

26. Sugar beet plant, exhibiting a resistance to BNYVV, consisting at least partly of plant cells as claimed in claims 17-23.

27. Progeny of a sugar beet plant as claimed in claim 26.

28. Seeds of a sugar beet plant as claimed in claim 26.

29. Vegetatively reproducible structures, such as calluses, buds, embryos, from a plant according to claim 26 or progeny according to claim 27.